STIC-Biotech/ChemLib 66477 CIRF

From: Lacourciere, Karen
Sent: Friday, May 10, 2002 3:18 PM
To: STIC-Biotech/ChemLib
Subject: Sequence search request 09/898,556

Please perform an **oligomer** search on SEQ ID NO:**3, 10 and 11** for SN **09/898,556**. I am looking for short oligonucleotides (less than 100 nucleotides) which are complementary to these sequences.

Thank-you!

Karen A. Lacourciere Ph.D.
CM1 11D09 GAU 1635
(703) 308-7523
mailbox 11E12

seq. 10 has 11,173 residues
cancelled, sent to Montreal
5/10/02 EST

Point of Contact:
Susan Hanley
Technical Info. Specialist
CM1 6B05 Tel: 305-4053

mic - 3,10,11

Searcher: _____
Phone: _____
Location: _____
Date Picked Up: _____
Date Completed: _____
Searcher Prep/Review: _____
Clerical: _____
Online time: _____

TYPE OF SEARCH:
NA Sequences: _____
AA Sequences: _____
Structures: _____
Bibliographic: _____
Litigation: _____
Full text: _____
Patent Family: _____
Other: _____

VENDOR/COST(where applic
STN: _____
DIALOG: _____
Questel/Orbit: _____
DRLink: _____
Lexis/Nexis: _____
Sequence Sys.: _____
WWW/Internet: _____
Other (specify): _____

FILE 'HOME' ENTERED AT 15:28:45 ON 10 MAY 2002

=> b medline caplus lifesci embase uspatfull biosis

COST IN U.S. DOLLARS	SINCE FILE ENTRY	TOTAL SESSION
FULL ESTIMATED COST	0.21	0.21

FILE 'MEDLINE' ENTERED AT 15:29:01 ON 10 MAY 2002

FILE 'CAPLUS' ENTERED AT 15:29:01 ON 10 MAY 2002
USE IS SUBJECT TO THE TERMS OF YOUR STN CUSTOMER AGREEMENT.
PLEASE SEE "HELP USAGETERMS" FOR DETAILS.

FILE 'LIFESCI' ENTERED AT 15:29:01 ON 10 MAY 2002

FILE 'EMBASE' ENTERED AT 15:29:01 ON 10 MAY 2002

FILE 'USPATFULL' ENTERED AT 15:29:01 ON 10 MAY 2002

FILE 'BIOSIS' ENTERED AT 15:29:01 ON 10 MAY 2002

=> s hkr1 or (hkr()1) or (gli()related finger protein) or (gli()kruppel family member) or (glioma associated oncogene)

L1 47 HKR1 OR (HKR(W) 1) OR (GLI(W) RELATED FINGER PROTEIN) OR (GLI(W) KRUPPEL FAMILY MEMBER) OR (GLIOMA ASSOCIATED ONCOGENE)

=> dup rem l1

PROCESSING COMPLETED FOR L1
L2 18 DUP REM L1 (29 DUPLICATES REMOVED)

=> s l2 and (antisens? or Ribozym? or triplex)

L3 5 L2 AND (ANTISENS? OR RIBOZYM? OR TRIPLEX)

=> d l3 ibib abs tot

L3 ANSWER 1 OF 5 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 2001:895610 CAPLUS
DOCUMENT NUMBER: 136:35877
TITLE: **Antisense** oligonucleotides to **glioma -associated oncogene**-1 and their use in diagnosis and treatment of cancer
INVENTOR(S): Bennett, C. Frank; Wyatt, Jacqueline
PATENT ASSIGNEE(S): Isis Pharmaceuticals, Inc., USA
SOURCE: U.S., 43 pp.
CODEN: USXXAM
DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 1

PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
US 6329203	B1	20011211	US 2000-657042	20000908
WO 2002020549	A1	20020314	WO 2001-US28082	20010907

W: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM

RW: GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW, AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG

PRIORITY APPLN. INFO.: US 2000-657042 A 20000908

AB **Antisense** compds., compns. and methods are provided for modulating the expression of glioma-assocd. oncogene-1. The compns. comprise **antisense** compds., particularly **antisense** oligonucleotides, targeted to nucleic acids encoding glioma-assocd. oncogene-1. Methods of using these compds. for modulation of glioma-assocd. oncogene-1 expression and for treatment of diseases assocd. with expression of glioma-assocd. oncogene-1 are provided. Pharmaceutical compns. for treatment of cancers assocd. with the central nervous system, skin and musculoskeletal system are provided.

REFERENCE COUNT: 15 THERE ARE 15 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

L3 ANSWER 2 OF 5 USPATFULL

ACCESSION NUMBER: 2001:79288 USPATFULL

TITLE: Genomic DNA sequences of ashbya gossypii and uses thereof

INVENTOR(S): Philippsen, Peter, Riehen, Switzerland
Pohlmann, Rainer, Lorrach, Germany, Federal Republic of
Steiner-Lange, Sabine, Bonn, Germany, Federal Republic of
Mohr, Christine, Allschwil, Switzerland
Wendland, Jurgen, Lorrach, Germany, Federal Republic of
Knechtle, Philipp, Oberwil, Switzerland
Rebischung, Corinne, Saint-Louis, France

PATENT ASSIGNEE(S): Syngenta Participations AG, Basel, Switzerland (non-U.S. corporation)

	NUMBER	KIND	DATE	
PATENT INFORMATION:	US 6239264	B1	20010529	
APPLICATION INFO.:	US 1997-998416		19971224	(8)

DOCUMENT TYPE: Utility
FILE SEGMENT: Granted
PRIMARY EXAMINER: Priebe, Scott D.
LEGAL REPRESENTATIVE: Meigs, J. Timothy
NUMBER OF CLAIMS: 2
EXEMPLARY CLAIM: 1
LINE COUNT: 4269
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB The present invention relates to the terminal sequencing of random genomic fragments performed with the filamentous fungus A.gossypii, to the sequences obtained therewith and the use of the sequences for forensic identification, to characterize genes and gene organization of this ascomycete by inter-genomic comparison, to identify biosynthetic

genes that can be used as selection markers, to isolate promotors and terminators for application in a homologous as well as heterologous context, to find putative centromere containing clones, chromosome mapping, chromosome identifying, general information about chromosome organization and in addition to identify ORF containing SRS sequences with no homology to S. cerevisiae or any other organism which allows the identification of A. gossypii specific genes.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L3 ANSWER 3 OF 5 USPATFULL

ACCESSION NUMBER:	2001:18599 USPATFULL
TITLE:	Method for modulating processes mediated by farnesoid activated receptors
INVENTOR(S):	Evans, Ronald M., La Jolla, CA, United States Forman, Barry M., La Jolla, CA, United States Weinberger, Cary A., Carrboro, NC, United States
PATENT ASSIGNEE(S):	The Salk Institute for Biological Studies, La Jolla, CA, United States (U.S. corporation)

	NUMBER	KIND	DATE
PATENT INFORMATION:	US 6184353	B1	20010206
APPLICATION INFO.:	US 1999-469721		19991221 (9)

RELATED APPLN. INFO.:	Continuation of Ser. No. US 1995-372183, filed on 13 Jan 1995, now patented, Pat. No. US 6005086
DOCUMENT TYPE:	Utility
FILE SEGMENT:	Granted
PRIMARY EXAMINER:	Carlson, Karen Cochrane
LEGAL REPRESENTATIVE:	Gray Cary Ware & Freidenrich LLP, Reiter, Stephen E., Stewart, Ramsey R.
NUMBER OF CLAIMS:	8
EXEMPLARY CLAIM:	1
NUMBER OF DRAWINGS:	10 Drawing Figure(s); 6 Drawing Page(s)
LINE COUNT:	881

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB Farnesyl pyrophosphate, the metabolically active form of farnesol, is a key precursor in the synthesis of cholesterol, carotenoids, steroid hormones, bile acids and other molecules involved in cellular growth and metabolism. A nuclear receptor has been identified that is transcriptionally activated by farnesol and related molecules. This novel signaling pathway can be modulated by the use of key metabolic intermediates (or analogs and/or derivatives thereof) as transcriptional regulatory signals.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L3 ANSWER 4 OF 5 USPATFULL

ACCESSION NUMBER:	1999:167126 USPATFULL
TITLE:	Farnesoid activated receptor polypeptides, and nucleic acid encoding the same
INVENTOR(S):	Evans, Ronald M., La Jolla, CA, United States Forman, Barry M., La Jolla, CA, United States Weinberger, Cary A., Carrboro, NC, United States
PATENT ASSIGNEE(S):	The Salk Institute for Biological Studies, La Jolla, CA, United States (U.S. corporation)

	NUMBER	KIND	DATE
PATENT INFORMATION:	US 6005086		19991221
APPLICATION INFO.:	US 1995-372183		19950113 (8)

DOCUMENT TYPE:	Utility
FILE SEGMENT:	Granted

PRIMARY EXAMINER: Carlson, Karen Cochrane
LEGAL REPRESENTATIVE: Gray Cary Ware & Freidenrich LLP, Reiter, Stephen E.
NUMBER OF CLAIMS: 10
EXEMPLARY CLAIM: 1
NUMBER OF DRAWINGS: 10 Drawing Figure(s); 6 Drawing Page(s)
LINE COUNT: 1023
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
AB Farnesyl pyrophosphate, the metabolically active form of farnesol, is a key precursor in the synthesis of cholesterol, carotenoids, steroid hormones, bile acids and other molecules involved in cellular growth and metabolism. A nuclear receptor has been identified that is transcriptionally activated by farnesol and related molecules. This novel signaling pathway can be modulated by the use of key metabolic intermediates (or analogs and/or derivatives thereof) as transcriptional regulatory signals.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L3 ANSWER 5 OF 5 USPATFULL
ACCESSION NUMBER: 97:68580 USPATFULL
TITLE: **Antisense** oligonucleotides to p53
INVENTOR(S): Smith, Larry James, Omaha, NE, United States
PATENT ASSIGNEE(S): University of Nebraska Board of Regents, Omaha, NE, United States (U.S. corporation)

	NUMBER	KIND	DATE
PATENT INFORMATION:	US 5654415		19970805
APPLICATION INFO.:	US 1994-327371		19941021 (8)

RELATED APPLN. INFO.: Division of Ser. No. US 1991-748997, filed on 23 Aug 1991, now abandoned
DOCUMENT TYPE: Utility
FILE SEGMENT: Granted
PRIMARY EXAMINER: Chambers, Jasemine C.
ASSISTANT EXAMINER: Crouch, Deborah
LEGAL REPRESENTATIVE: Dann, Dorfman, Herrell and Skillman
NUMBER OF CLAIMS: 3
EXEMPLARY CLAIM: 1
NUMBER OF DRAWINGS: 1 Drawing Figure(s); 1 Drawing Page(s)
LINE COUNT: 1618
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
AB **Antisense** oligonucleotides and composition of oligonucleotides are taught which inhibit the proliferation of cells without affecting cell viability. p53 **antisense** oligonucleotides are shown to inhibit the proliferation of tissue culture cells expressing this gene. Methods of treating a patient with such **antisense** oligonucleotides is described.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

=> d l3 ibib kwic tot

L3 ANSWER 1 OF 5 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 2001:895610 CAPLUS
DOCUMENT NUMBER: 136:35877
TITLE: **Antisense** oligonucleotides to **glioma -associated oncogene**-1 and their use in diagnosis and treatment of cancer
INVENTOR(S): Bennett, C. Frank; Wyatt, Jacqueline
PATENT ASSIGNEE(S): Isis Pharmaceuticals, Inc., USA
SOURCE: U.S., 43 pp.
CODEN: USXXAM

DOCUMENT TYPE: Patent
LANGUAGE: English
FAMILY ACC. NUM. COUNT: 1
PATENT INFORMATION:

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
US 6329203	B1	20011211	US 2000-657042	20000908
WO 2002020549	A1	20020314	WO 2001-US28082	20010907

W: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM

RW: GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW, AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG

PRIORITY APPLN. INFO.: US 2000-657042 A 20000908
REFERENCE COUNT: 15 THERE ARE 15 CITED REFERENCES AVAILABLE FOR THIS
RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT

TI **Antisense** oligonucleotides to **glioma-associated oncogene**-1 and their use in diagnosis and treatment of cancer

AB **Antisense** compds., compns. and methods are provided for modulating the expression of glioma-assocd. oncogene-1. The compns. comprise **antisense** compds., particularly **antisense** oligonucleotides, targeted to nucleic acids encoding glioma-assocd. oncogene-1. Methods of using these compds. for modulation of glioma-assocd. oncogene-1 expression and for treatment of diseases assocd. with expression of glioma-assocd. oncogene-1 are provided. Pharmaceutical compns. for treatment of cancers assocd. with the central nervous system, skin and musculoskeletal system are provided.

ST **antisense** DNA glioma assocd oncogene 1 diagnosis treatment cancer

IT Oligonucleotides
Phosphorothioate oligonucleotides
RL: PRP (Properties); THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(2'-O-methoxyethyl sugar or 5-methylcytosine in; **antisense** oligonucleotides to glioma-assocd. oncogene-1 and their use in diagnosis and treatment of cancer)

IT Neoplasm
(**antisense** oligonucleotides to glioma-assocd. oncogene-1 and their use in diagnosis and treatment of cancer)

IT Drug delivery systems
(colloidal dispersion, **antisense** DNA to glioma-assocd. oncogene-1 in; **antisense** oligonucleotides to glioma-assocd. oncogene-1 and their use in diagnosis and treatment of cancer)

IT mRNA
RL: BSU (Biological study, unclassified); THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(glioma assoc. oncogene-1; **antisense** oligonucleotides to glioma-assocd. oncogene-1 and their use in diagnosis and treatment of cancer)

IT Neuroglia
(glioma; **antisense** oligonucleotides to glioma-assocd. oncogene-1 and their use in diagnosis and treatment of cancer)

IT Gene, animal
RL: BSU (Biological study, unclassified); PRP (Properties); THU (Therapeutic use); BIOL (Biological study); USES (Uses)

(oncogene, glioma assoc. oncogene-1; **antisense** oligonucleotides to glioma-assocd. oncogene-1 and their use in diagnosis and treatment of cancer)

IT **Antisense** DNA
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(to glioma-assocd. oncogene-1, contg. modified sugar or nucleobase moiety; **antisense** oligonucleotides to glioma-assocd. oncogene-1 and their use in diagnosis and treatment of cancer)

IT 288716-75-6
RL: PRP (Properties)
(Unclaimed; **antisense** oligonucleotides to glioma-assocd. oncogene-1 and their use in diagnosis and treatment of cancer)

IT 360154-20-7 380503-80-0 380503-81-1 380503-82-2 380503-83-3
380503-84-4 380503-85-5 380503-86-6 380503-87-7 380503-88-8
380503-89-9 380503-90-2 380503-91-3 380503-92-4 380503-93-5
380503-94-6 380503-95-7 380503-96-8 380503-97-9 380503-98-0
380503-99-1 380504-00-7 380504-01-8 380504-02-9 380504-03-0
380504-04-1 380504-05-2 380504-06-3 380504-07-4 380504-08-5
380504-09-6 380504-10-9 380504-11-0 380504-12-1 380504-13-2
380504-14-3 380504-15-4 380504-16-5 380504-17-6 380504-18-7
380504-19-8 380504-20-1 380504-21-2 380504-22-3 380504-23-4
380504-24-5 380504-25-6 380504-26-7 380504-27-8 380504-28-9
380504-29-0 380504-30-3 380504-31-4 380504-32-5 380504-33-6
380504-34-7 380504-35-8 380504-36-9 380504-37-0 380504-38-1
380504-39-2 380504-40-5 380504-41-6 380504-42-7
RL: PRP (Properties); THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(glioma-assocd. oncogene-1 **antisense** DNA; **antisense** oligonucleotides to glioma-assocd. oncogene-1 and their use in diagnosis and treatment of cancer)

IT 554-01-8, 5-Methylcytosine
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(in **antisense** DNAs; **antisense** oligonucleotides to glioma-assocd. oncogene-1 and their use in diagnosis and treatment of cancer)

IT 140279-09-0 181982-21-8, 2: PN: WO0004034 SEQID: 2 unclaimed DNA
206225-09-4 343902-48-7 343902-49-8 343902-50-1 380504-43-8
380504-44-9 380504-45-0 380504-46-1 380504-47-2 380504-48-3
380504-49-4 380504-50-7 380504-51-8 380504-52-9 380504-53-0
380504-54-1 380504-55-2 380504-56-3 380504-57-4 380504-58-5
380504-59-6
RL: PRP (Properties)
(unclaimed nucleotide sequence; **antisense** oligonucleotides to glioma-assocd. oncogene-1 and their use in diagnosis and treatment of cancer)

L3 ANSWER 2 OF 5 USPATFULL
ACCESSION NUMBER: 2001:79288 USPATFULL
TITLE: Genomic DNA sequences of ashbya gossypii and uses thereof
INVENTOR(S): Philippsen, Peter, Riehen, Switzerland
Pohlmann, Rainer, Lorrach, Germany, Federal Republic of
Steiner-Lange, Sabine, Bonn, Germany, Federal Republic of
Mohr, Christine, Allschwil, Switzerland
Wendland, Jurgen, Lorrach, Germany, Federal Republic of
Knechtle, Philipp, Oberwil, Switzerland
Rebischung, Corinne, Saint-Louis, France
PATENT ASSIGNEE(S): Syngenta Participations AG, Basel, Switzerland (non-U.S. corporation)

NUMBER	KIND	DATE

PATENT INFORMATION: US 6239264 B1 20010529
APPLICATION INFO.: US [illegible]97-998416 19971224 (8)
DOCUMENT TYPE: Uti[illegible]y
FILE SEGMENT: Granted
PRIMARY EXAMINER: Priebe, Scott D.
LEGAL REPRESENTATIVE: Meigs, J. Timothy
NUMBER OF CLAIMS: 2
EXEMPLARY CLAIM: 1
LINE COUNT: 4269
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
DETD 1.) Generation of **antisense** transcripts.
DETD . . . mating-type switching; mutant
cells die if mating-type switching is attempted, mutants are unable to repair double-strand breaks

PAG1586RP	tRNA-Val		1
PAG1586UP	YDR420w	**HKR1**	Hansenula mrakii K9 killer toxin-resistance 2 protein
PAG1587RP	YAL036c	FUN11	YAL036c:unknown, has GTP-binding motif 1
PAG1587UP	YOR346w	REV1	protein required for mutagenesis. . .

L3 ANSWER 3 OF 5 USPATFULL
ACCESSION NUMBER: 2001:18599 USPATFULL
TITLE: Method for modulating processes mediated by farnesoid activated receptors
INVENTOR(S): Evans, Ronald M., La Jolla, CA, United States
Forman, Barry M., La Jolla, CA, United States
Weinberger, Cary A., Carrboro, NC, United States
PATENT ASSIGNEE(S): The Salk Institute for Biological Studies, La Jolla, CA, United States (U.S. corporation)

	NUMBER	KIND	DATE
PATENT INFORMATION:	US 6184353	B1	20010206
APPLICATION INFO.:	US 1999-469721		19991221 (9)

RELATED APPLN. INFO.: Continuation of Ser. No. US 1995-372183, filed on 13 Jan 1995, now patented, Pat. No. US 6005086
DOCUMENT TYPE: Utility
FILE SEGMENT: Granted
PRIMARY EXAMINER: Carlson, Karen Cochrane
LEGAL REPRESENTATIVE: Gray Cary Ware & Freidenrich LLP, Reiter, Stephen E., Stewart, Ramsey R.
NUMBER OF CLAIMS: 8
EXEMPLARY CLAIM: 1
NUMBER OF DRAWINGS: 10 Drawing Figure(s); 6 Drawing Page(s)
LINE COUNT: 881
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
DETD In situ hybridization/histochemistry was performed to further localize sites of FAR expression. **Antisense** cRNA probes from truncated mouse FAR cDNA or full-length mouse RXR.beta. cDNA were used. The control was a truncated rat. . .
DETD . . . 700 markers including the Chr 10 markers Pfp (pore forming protein), Tral (tumor rejection antigen gp96), Ifg (interferon .gamma.),
Gli (**glioma associated oncogene**) and
Gad1-ps1 (glutamic acid decarboxylase 1 pseudogene).

L3 ANSWER 4 OF 5 USPATFULL
ACCESSION NUMBER: 1999:167126 USPATFULL
TITLE: Farnesoid activated receptor polypeptides, and nucleic acid encoding the same
INVENTOR(S): Evans, Ronald M., La Jolla, CA, United States
Forman, Barry M., La Jolla, CA, United States
Weinberger, Cary A., Carrboro, NC, United States
PATENT ASSIGNEE(S): The Salk Institute for Biological Studies, La Jolla,

CA, United States (U.S. corporation)

	NUMBER	KIND	DATE
PATENT INFORMATION:	US 6005086		19991221
APPLICATION INFO.:	US 1995-372183		19950113 (8)

DOCUMENT TYPE: Utility
FILE SEGMENT: Granted
PRIMARY EXAMINER: Carlson, Karen Cochrane
LEGAL REPRESENTATIVE: Gray Cary Ware & Freidenrich LLP, Reiter, Stephen E.
NUMBER OF CLAIMS: 10
EXEMPLARY CLAIM: 1
NUMBER OF DRAWINGS: 10 Drawing Figure(s); 6 Drawing Page(s)
LINE COUNT: 1023
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
DETD In situ hybridization/histochemistry was performed to further localize sites of FAR expression. **Antisense** cRNA probes from truncated mouse FAR cDNA or full-length mouse RXR.beta. cDNA were used. The control was a truncated rat. . .
DETD . . . 700 markers including the Chr 10 markers Pfp (pore forming protein), Tral (tumor rejection antigen gp96), Ifg (interferon .gamma.), Gli (**glioma associated oncogene**) and Gad1-ps1 (glutamic acid decarboxylase 1 pseudogene).

L3 ANSWER 5 OF 5 USPATFULL
ACCESSION NUMBER: 97:68580 USPATFULL
TITLE: **Antisense** oligonucleotides to p53
INVENTOR(S): Smith, Larry James, Omaha, NE, United States
PATENT ASSIGNEE(S): University of Nebraska Board of Regents, Omaha, NE, United States (U.S. corporation)

	NUMBER	KIND	DATE
PATENT INFORMATION:	US 5654415		19970805
APPLICATION INFO.:	US 1994-327371		19941021 (8)

RELATED APPLN. INFO.: Division of Ser. No. US 1991-748997, filed on 23 Aug 1991, now abandoned
DOCUMENT TYPE: Utility
FILE SEGMENT: Granted
PRIMARY EXAMINER: Chambers, Jasemine C.
ASSISTANT EXAMINER: Crouch, Deborah
LEGAL REPRESENTATIVE: Dann, Dorfman, Herrell and Skillman
NUMBER OF CLAIMS: 3
EXEMPLARY CLAIM: 1
NUMBER OF DRAWINGS: 1 Drawing Figure(s); 1 Drawing Page(s)
LINE COUNT: 1618
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
TI **Antisense** oligonucleotides to p53
AB **Antisense** oligonucleotides and composition of oligonucleotides are taught which inhibit the proliferation of cells without affecting cell viability. p53 **antisense** oligonucleotides are shown to inhibit the proliferation of tissue culture cells expressing this gene. Methods of treating a patient with such **antisense** oligonucleotides is described.
SUMM Very recent studies involving the use of **antisense** oligonucleotides for treatment of cancer have been reviewed by Stein and Cohen, Cancer Res. 48:2659 (1988). Several types of **antisense** molecules have been screened for their ability to inhibit the synthesis of particular proteins using both intact cells and in. . .
SUMM . . . causing genes. They give blocking of NF-kB binding to HIV enhancer as an example. The use of retroviral vectors carrying **antisense** oncogenes for the treatment of cancer is known.
SUMM In the case of cancer, contemplated therapy involving **antisense** expression vector ODNs have been directed to oncogenes in accordance

with the oncogene/anti-oncogene cancer model, or to growth factors expressed. . .

SUMM . . . effective a[redacted]nt of a composition selected f[redacted]n the group consisting of an expression vector, a double stranded ODN, and an **antisense** ODN. Said composition must be capable of regulating expression of a TR. Said TR is expressed by the AP cells. . .

SUMM . . . said individual an effective amount of a composition selected from the group consisting of a double stranded ODN and an **antisense** ODN. The composition is capable of regulating expression of a TR. The TR is expressed by therapeutically relevant cells and. . .

SUMM . . . embodiment of this invention. This method involves removing and

culturing AP disease cells from an AP disease patient with an **antisense** ODN specific to a TR from the relevant subset of TRs expressed by AP cells from an AP patient or. . .

SUMM . . . but not in tissue where side effects are produced by destruction of said RNA. Exemplary is the use of an **antisense** ODN directed to cyclooxygenase RNA that selectively binds to and destroys said RNA in hematopoietic tissue while avoiding said RNA. .

.

DETD The present inventor has found that **antisense** p53 oligonucleotides can inhibit the proliferation, including the blocking of stem cell self-renewal, and ultimately kill primary human leukemic blasts. . . interpretation) that the inhibition of a single gene or set of genes coding for proteins involved in this process by **antisense** oligonucleotides is sufficient to change the impact of the informational molecules so a change in cellular programming such as cellular. . . genes" is used herein to describe those genes in AP cells that may be suitable for targeting for inhibition with **antisense** molecules in accordance with the present invention Suitable target or traitor genes may themselves either be functionally abnormal or be. . .

DETD . . . of factors, including the type of cancerous cells present in the marrow, the type, and the specificity of the particular **antisense** oligonucleotide(s) selected, and the relative toxicity of the oligonucleotide for normal cells. Although the present inventor has observed significant AP. . .

DETD a) **Antisense** oligonucleotides (Zon, Pharmaceut. Res., 5, 539, 1988).

DETD Design of "test" **antisense** oligonucleotides

DETD . . . Using a computer program such as "Oligo" (Rychik and Rhoads, Nucl. Acids Res., 17., 8543, 1989) select a set of **antisense** oligonucleotides that bind to the RNA target of choice that have the following characteristics: (1). length between 10 and 35. . .

DETD ii) Using a reference such as Genbank ensure that the **antisense** oligonucleotide has .ltoreq.85% homology with the RNA transcripts of other genes. An exception to this is where an **antisense** oligonucleotide is selected on the basis of its ability to bind to more than one member of a transcriptional regulator. . .

DETD b) Establishment of "prototype therapeutic" **antisense** oligonucleotide from a set of test **antisense** oligonucleotides. These prototype compounds will be used in the reduction to practice.

DETD i) Synthesize test **antisense** oligonucleotides using standard procedures, for example, those for producing phosphorothioates (Vu et al, Tetrahedron Lett, 32, 3005, 1991).

DETD ii) Using assays for transcriptional regulators or their direct modifiers select prototype therapeutic **antisense** oligonucleotides out of the set of test compounds on the basis of shutting down expression of the target gene in. . .

DETD . . . be constructed and tested using standard methods. (Ausubel et al, supra) Alternatively, the viral vector will carry a sufficiently long **antisense** sequence to such a regulator or modifier to provide for the blocking of expression of the target gene in the. . .

DETD i) can be achieved by the use of **antisense** oligonucleotides directed to the RNA of a particular transcriptional regulator or direct

modulator or double-stranded oligonucleotide ligands for DNA binding.

.

. .

DETD Using prototype **antisense** oligonucleotide(s) or double-stranded oligonucleotides block function of specific transcriptional regulator(s) in aberrantly programmed cells or normal cells to be therapeutically manipulated through reprogramming. Alternatively use an **antisense** oligonucleotide directed to a direct modifier of a transcriptional regulator.

DETD ii) Using expression vector carrying **antisense** DNA directed to a particular transcriptional regulator or a direct modifier of a transcriptional regulator, install the new gene in aberrantly programmed cells. The therapeutic effect will be determined in advance through the use of an **antisense** oligonucleotide.

DETD a) **Antisense** oligonucleotides

DETD FIG. 1 demonstrates that there are cell type specific differences in effects of particular **antisense** oligonucleotides targeted to different sites on specific RNA transcripts on cell behavior. Such differences can be used to select **antisense** oligonucleotides that produce the desired therapeutic effects with minimal undesirable side effects.

DETD A set of four different phosphorothioate **antisense** oligonucleotides directed to p53. RNA were prepared using an Applied Biosystems, Inc. (ABI) DNA synthesizer (Model 380B) according to the manufacturer's protocols. An **antisense** oligonucleotide against the HIV rev gene was used as a negative control. The sequences are set forth in the Sequence. . . normal adult human gastrointestinal epithelium, normal human fetal gastrointestinal epithelium and Rhesus monkey T-lymphocytes. Destruction of p53 RNA by the **antisense** p53 oligonucleotides was documented using PCR and/or dot blotting.

DETD The following effects of the **antisense** p53 oligonucleotides on cellular programming were evident from the results found.

DETD 3) The data also demonstrates that there are cell type specific differences in responses to **antisense** oligonucleotides targeted to different sites on RNA transcripts of the same gene (FIG. 1). This provides a basis for optimizing. . .

DETD 4) These results support the general principle that **antisense** oligonucleotides directed to transcriptional regulator can be used to expand particular normal adult or fetal tissues vitro that could then. . .

DETD 5) The cell type dependency of the effects of particular **antisense** oligonucleotides directed to a transcriptional regulator support the cellular program model in general and the aberrant program model in particular.

DETD The ability of the **antisense** p53 oligonucleotides to recognize the p53 RNA of Rhesus monkeys was demonstrated by showing a similar inhibitory effect on mature. . .

DETD Two Rhesus monkeys weighing 8.9 kg and 6.8 kg were infused with 52.5 mg and 75.8 mg of the OL(1)p53 **antisense** oligonucleotide (SEQ ID NO:4) which was radiolabelled over four hours. In keeping with rodent data, tissue distribution analysis showed substantial. . .

DETD Since no unacceptable side effects were produced in the monkeys, it has not been necessary to modify the **antisense** oligonucleotides.

DETD The **antisense** oligonucleotide selected for practice of the invention may be any of the types described by Stein and Cohen, Cancer Research. . . unmodified oligodeoxynucleotides, ethyl- or methyl-phosphonate modified oligodeoxynucleotides, phosphorothioate modified oligonucleotides, dithioates, as well as other oligonucleotide analogs, including those incorporating **ribozyme** structures, and oligoribonucleotides such as those described by Inove et al., Nucleic Acids Res. 5:6131 (1987); and Chimeric oligonucleotides that.

.

. RNA, DNA analogues (Inove, et al, FEBS Lett. 115:327 (1987). Oligonucleotides having a lipophilic backbone, for example,

methylphosphonate analogs with **ribozyme** structures, may prove advantageous in cert circumstances; these molecules may have a longer
half-life in vivo since the lipophilic structure may reduce the rate of renal clearance while the **ribozyme** structure promotes cleavage of the target RNA. Gerlach, Nature 334:585 (1988).
DETD In addition to the **antisense** oligonucleotide compounds, the pharmaceutical compositions of the invention may contain any of a number
of suitable excipients and auxiliaries which. . .
DETD . . . the essential nature of the model of clinical cancer given in the patent application and the basic rationale for using **antisense** oligonucleotides directed against the indicated target or traitor genes as therapeutic agents. It should be clear that this is a. . .
DETD II. ANALOGY WITH **ANTISENSE** OLIGONUCLEOTIDE TREATMENT STRATEGY
DETD . . . will kill low grade 1 and 2 and high grade 1, but it also kills
normal cell type 1. So **antisense** inhibition of "a" might be useful for purging bone marrow of malignant cell type 1 or 2 but not for. . .
DETD . . . liver, astocytes

3723, 1990.

GLI-1-3 GLI embryonal carcinoma,
Ruppert, et al., Mol.
-- --
myometrium testis,
Cell. Biol. 8, 3104,
placenta 1988.
HKR1-4 HKR testis, placenta,
Ruppert, et al., Mol.
-- --
kidney, colon, lung,
Cell. Biol. 8, 3104,
brain, embryonal
1988.
carcinoma
HOX. . .

=> d history

(FILE 'HOME' ENTERED AT 15:28:45 ON 10 MAY 2002)

FILE 'MEDLINE, CAPLUS, LIFESCI, EMBASE, USPATFULL, BIOSIS' ENTERED AT 15:29:01 ON 10 MAY 2002
L1 47 S HKR1 OR (HKR()1) OR (GLI()RELATED FINGER PROTEIN) OR (GLI()KR
L2 18 DUP REM L1 (29 DUPLICATES REMOVED)
L3 5 S L2 AND (ANTISENS? OR RIBOZYM? OR TRIPLEX)

=> s l2 not l3

L4 13 L2 NOT L3

=> d l4 ibib abs tot

L4 ANSWER 1 OF 13 MEDLINE
ACCESSION NUMBER: 2001690825 MEDLINE
DOCUMENT NUMBER: 21599055 PubMed ID: 11736809
TITLE: An updated linkage and comparative map of porcine chromosome 18.
AUTHOR: Campbell E M; Fahrenkrug S C; Vallet J L; Smith T P; Rohrer .

G A
CORPORATE SOURCE: USDA, ARS, US Meat Animal Research Center, PO Box 166, Spur
18D, Clay Center, NE 68933-0166, USA.
SOURCE: ANIMAL GENETICS, (2001 Dec) 32 (6) 375-9.
Journal code: 8605704. ISSN: 0268-9146.
PUB. COUNTRY: England: United Kingdom
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 200202
ENTRY DATE: Entered STN: 20011213
Last Updated on STN: 20020215
Entered Medline: 20020214

AB Swine chromosome 18 (SSC18) has the poorest marker density in the USDA-MARC porcine linkage map. In order to increase the marker density, seven genes from human chromosome 7 (HSA7) expected to map to SSC18 were selected for marker development. The genes selected were: growth hormone releasing hormone receptor (GHRHR), **GLI-Kruppel family member** (GLI3), leptin (LEP), capping protein muscle Z-line alpha 2 subunit (CAPZA2), beta A inhibin (INHBA), T-cell receptor beta (TCRB) and T-cell receptor gamma (TCRG). Large-insert clones (YACs, BACs and cosmids) that contained these genes, as well as two previously mapped microsatellite markers (SW1808 and SW1984), were identified and screened for microsatellites. New microsatellite markers were developed from these clones and mapped. Selected clones were also physically assigned by fluorescence in situ hybridization (FISH). Fifteen new microsatellite markers were added to the SSC18 linkage map resulting in a map of 28 markers. Six genes have been included into the genetic map improving the resolution of the SSC18 and HSA7 comparative map. Assignment of TCRG to SSC9 has identified a break in conserved synteny between SSC18 and HSA7.

L4 ANSWER 2 OF 13 MEDLINE
ACCESSION NUMBER: 2000033541 MEDLINE
DOCUMENT NUMBER: 20033541 PubMed ID: 10564805
TITLE: Variations in mRNA transcript levels of cell wall-associated genes of Saccharomyces cerevisiae following spheroplasting.
AUTHOR: Braley R; Chaffin W L
CORPORATE SOURCE: Department of Microbiology and Immunology, Texas Tech University Health Sciences Center, Lubbock, TX 79430, USA.
CONTRACT NUMBER: AI23416 (NIAID)
SOURCE: FEMS MICROBIOLOGY LETTERS, (1999 Dec 1) 181 (1) 177-85.
Journal code: FML; 7705721. ISSN: 0378-1097.
PUB. COUNTRY: Netherlands
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 200002
ENTRY DATE: Entered STN: 20000209
Last Updated on STN: 20000209
Entered Medline: 20000203

AB mRNA transcript levels of 38 genes from Saccharomyces cerevisiae were investigated during attempted spheroplast regeneration. Many of the genes selected are involved in cell wall biosynthesis. Spheroplasts did not regenerate into osmotically competent cells during the experiment. However, at a mRNA level, the quantities of transcripts were altered between the experimental and control populations. KRE11, EGT2 and MSS10 had their transcript levels increased by more than 10-fold during attempted spheroplast regeneration. A further six genes, FLO1, TIR1, SED1,

HKR1, YGR189 and MUC1, showed transcript level increases of at least 5-fold. Five gen[redacted] showed a change in transcript levels from an undetectable level to [redacted]detectable level: SKT5, KRE1, K[redacted], SEC53 and DHS1. PMT2 showed a rapid decrease in mRNA levels followed by an increase to the basal level. Thus, cell stress genes, biosynthetic genes and some glycosylphosphatidylinositol-anchored cell wall proteins have their transcript levels increased in regenerating spheroplasts, but their transcription was not sufficient to initiate the replacement of the cell wall in liquid medium.

L4 ANSWER 3 OF 13 MEDLINE
ACCESSION NUMBER: 1999200485 MEDLINE
DOCUMENT NUMBER: 99200485 PubMed ID: 10102362
TITLE: Genetic analysis of mutant clones of Chlamydomonas reinhardtii defective in potassium transport.
AUTHOR: Polley L D
CORPORATE SOURCE: Wabash College, Department of Biology Crawfordsville, IN 47933, USA.
SOURCE: MOLECULAR AND GENERAL GENETICS, (1999 Mar) 261 (2) 275-80. Journal code: NGP; 0125036. ISSN: 0026-8925.
PUB. COUNTRY: GERMANY: Germany, Federal Republic of
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199904
ENTRY DATE: Entered STN: 19990511
Last Updated on STN: 19990511
Entered Medline: 19990426

AB Mutant clones of Chlamydomonas reinhardtii defective for potassium transport were isolated and characterized. Of the four genes identified, three - TRK1, TRK2 and TRK3 encode high-affinity transport functions, and one gene, **HKR1**, encodes a low-affinity transport function. Characterization of the potassium dependence of recombinants possessing two mutant trk alleles suggests that the protein products of TRK2 and TRK3 interact functionally, and that TRK1 may serve a regulatory function. The mutant clone defective for a low-affinity potassium transporter was isolated by mutagenizing trk2-1 cells, which lack a functional high-affinity transporter, and screening surviving cells for dependence on very high potassium concentrations. The **hkr1** phenotype is expressed only in the presence of trk2-1.

L4 ANSWER 4 OF 13 MEDLINE
ACCESSION NUMBER: 1999033001 MEDLINE
DOCUMENT NUMBER: 99033001 PubMed ID: 9813242
TITLE: The Kruppel-type zinc finger family gene, **HKR1**, is induced in lung cancer by exposure to platinum drugs.
AUTHOR: Oguri T; Katoh O; Takahashi T; Isobe T; Kuramoto K; Hirata S; Yamakido M; Watanabe H
CORPORATE SOURCE: Second Department of Internal Medicine, Hiroshima University School of Medicine, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8551, Japan.
SOURCE: GENE, (1998 Nov 5) 222 (1) 61-7. Journal code: FOP; 7706761. ISSN: 0378-1119.
PUB. COUNTRY: Netherlands
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199901
ENTRY DATE: Entered STN: 19990202
Last Updated on STN: 19990202
Entered Medline: 19990115

AB To investigate the molecular mechanism associated with the signaling pathway of platinum drug administration, we focused on the C2H2-type zinc finger (ZNF) transcription factor gene family. Here we show cloning of a

Kruppel-type ZNF gene, **HKR1**, which contains Kruppel-associated box (KRAB) domain and ZnF motifs. We found that mRNA expression of the **HKR1** gene was induced in lung-cancer cell lines by exposure to cisplatin using Northern blot analysis. Moreover, we also found that **HKR1** mRNA expression levels in lung cancers were higher than those in normal lung tissues, and that high expression levels in lung cancers were associated with antemortem platinum drug administration. These results suggest that **HKR1** may be associated with the regulation of a signaling pathway involved in the progression of lung cancer or the acquisition of resistance to platinum drugs.

L4 ANSWER 5 OF 13 MEDLINE
ACCESSION NUMBER: 96134982 MEDLINE
DOCUMENT NUMBER: 96134982 PubMed ID: 8550469
TITLE: **HKR1** encodes a cell surface protein that regulates both cell wall beta-glucan synthesis and budding pattern in the yeast Saccharomyces cerevisiae.
AUTHOR: Yabe T; Yamada-Okabe T; Kasahara S; Furuichi Y; Nakajima T;
Ichishima E; Arisawa M; Yamada-Okabe H
CORPORATE SOURCE: Department of Mycology, Nippon Roche Research Center, Kamakura, Japan.
SOURCE: JOURNAL OF BACTERIOLOGY, (1996 Jan) 178 (2) 477-83.
Journal code: HH3; 2985120R. ISSN: 0021-9193.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199602
ENTRY DATE: Entered STN: 19960306
Last Updated on STN: 19960306
Entered Medline: 19960220

AB We previously isolated the Saccharomyces cerevisiae **HKR1** gene that confers on S. cerevisiae cells resistance to HM-1 killer toxin secreted by Hansenula mrakii (S. Kasahara, H. Yamada, T. Mio, Y. Shiratori, C. Miyamoto, T. Yabe, T. Nakajima, E. Ichishima, and Y. Furuichi, J. Bacteriol. 176:1488-1499, 1994). **HKR1** encodes a type 1 membrane protein that contains a calcium-binding consensus sequence (EF hand motif) in the cytoplasmic domain. Although the null mutation of **HKR1** is lethal, disruption of the 3' part of the coding region, which would result in deletion of the cytoplasmic domain of Hkr1p, did not affect the viability of yeast cells. This partial disruption of **HKR1** significantly reduced beta-1,3-glucan synthase activity and the amount of beta-1,3-glucan in the cell wall and altered the axial budding pattern of haploid cells. Neither chitin synthase activity nor chitin content was significantly affected in the cells harboring the partially disrupted **HKR1** allele. Immunofluorescence microscopy with an antibody raised against Hkr1p expressed in Escherichia coli revealed that Hkr1p was predominantly localized on the cell surface. The cell surface localization of Hkr1p required the N-terminal signal sequence because the C-terminal half of Hkr1p was detected uniformly in the cells. These results demonstrate that **HKR1** encodes a cell surface protein that regulates both cell wall beta-glucan synthesis and budding pattern and suggest that bud site assembly is somehow related to beta-glucan synthesis in S. cerevisiae.

L4 ANSWER 6 OF 13 MEDLINE
ACCESSION NUMBER: 94156857 MEDLINE
DOCUMENT NUMBER: 94156857 PubMed ID: 8113191
TITLE: Cloning of the Saccharomyces cerevisiae gene whose overexpression overcomes the effects of HM-1 killer toxin, which inhibits beta-glucan synthesis.
AUTHOR: Kasahara S; Yamada H; Mio T; Shiratori Y; Miyamoto C; Yabe

T; Nakajima T; Ichishima E; Furuichi Y
CORPORATE SOURCE: Department of Applied Biological Chemistry, Faculty of Agriculture, Tohoku University, Sendai, Japan.
SOURCE: JOURNAL OF BACTERIOLOGY, (1994 Mar) 176 (5) 1488-99.
Journal code: HH3; 2985120R. ISSN: 0021-9193.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
OTHER SOURCE: GENBANK-S69101
ENTRY MONTH: 199403
ENTRY DATE: Entered STN: 19940406
Last Updated on STN: 19970203
Entered Medline: 19940330

AB A gene whose overexpression can endow Saccharomyces cerevisiae cells with resistance to HM-1 killer toxin was cloned from an S. cerevisiae genomic library. This gene, designated **HKR1** (Hansenula mrakii killer toxin-resistant gene 1), contains a 5.4-kb open reading frame. The predicted amino acid sequence of the protein specified by **HKR1** indicates that the protein consists of 1,802 amino acids and is very rich in serine and threonine, which could serve as O-glycosylation sites. The protein also contains two hydrophobic domains at the N-terminal end and in the C-terminal half, which could function as a signal peptide and transmembrane domain, respectively. Hkr1p is found to contain an EF hand motif of the calcium-binding consensus sequence in the C-terminal cytoplasmic domain. Thus, Hkr1p is expected to be a calcium-binding, glycosylated type I membrane protein. Southern and Northern (RNA) analyses demonstrated that there is a single copy of the **HKR1** gene in the S. cerevisiae genome, and the transcriptional level of **HKR1** is extremely low. Gene disruption followed by tetrad analysis showed that **HKR1** is an essential gene. Overexpression of the truncated **HKR1** encoding the C-terminal half of Hkr1p made the cells more resistant to HM-1 killer toxin than the full-length **HKR1** did, demonstrating that the C-terminal half of Hkr1p is essential for overcoming the effect of HM-1 killer toxin. Furthermore, overexpression of **HKR1** increased the beta-glucan content in the cell wall without affecting in vitro beta-glucan synthase activity, suggesting that **HKR1** regulates beta-glucan synthesis in vivo.

L4 ANSWER 7 OF 13 MEDLINE
ACCESSION NUMBER: 91139122 MEDLINE
DOCUMENT NUMBER: 91139122 PubMed ID: 1981054
TITLE: A molecular genetic linkage map of mouse chromosome 7.
AUTHOR: Saunders A M; Seldin M F
CORPORATE SOURCE: Department of Medicine, Duke University Medical Center, Durham, North Carolina 27710.
CONTRACT NUMBER: HG00101 (NHGRI)
NS19999 (NINDS)
SOURCE: GENOMICS, (1990 Nov) 8 (3) 525-35.
Journal code: GEN; 8800135. ISSN: 0888-7543.
PUB. COUNTRY: United States
Journal; Article; (JOURNAL ARTICLE)
LANGUAGE: English
FILE SEGMENT: Priority Journals
ENTRY MONTH: 199103
ENTRY DATE: Entered STN: 19910412
Last Updated on STN: 20000303
Entered Medline: 19910325

AB The homology between mouse chromosome 7 and human chromosomes 11, 15, and 19 was examined using interspecific backcross animals derived from mating C3H/HeJ-gld/gld and Mus spretus mice. In an earlier study, we reported on the linkage relationships of 16 loci on mouse chromosome 7 and the homologous relationship between this chromosome and the myotonic dystrophy

gene region on human chromosome 19. Segregation analyses were used to extend the gene linkage relationships on mouse chromosome 7 by an additional 21 loci. Seven of these genes (Cyp2a, D19F11[redacted], Myod-1, Otf-2, Rnu1p70, Rnu2pa, and Xrcc-1) were previously unmapped in the mouse. Several potential mouse chromosome 7 genes (Mel, **Hkr-1**, Icam-1, Pvs) did not segregate with chromosome 7 markers, and provisional chromosomal assignments were made. This study establishes a detailed molecular genetic linkage map of mouse chromosome 7 that will be useful as a framework for determining linkage relationships of additional molecular markers and for identifying homologous disease genes in mice and humans.

L4 ANSWER 8 OF 13 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 2001:93856 CAPLUS
DOCUMENT NUMBER: 135:238195
TITLE: Expression analysis of zinc finger protein **HKR1** in neuroglioma
AUTHOR(S): Xia, Fang; Qi, Zhen-yu; Dai, Jian-liang; Gu, Shao-hua; Ma, Liang-xiao; Xie, Yi; Mao, Yu-min
CORPORATE SOURCE: Institute of Genetics, School of Life Sciences, Fudan University, Shanghai, 200433, Peop. Rep. China
SOURCE: Fudan Xuebao, Ziran Kexueban (2000), 39(6), 692-694
CODEN: FHPTAY; ISSN: 0427-7104
PUBLISHER: Fudan Daxue Chubanshe
DOCUMENT TYPE: Journal
LANGUAGE: Chinese
AB The cDNA of **HKR1** (Human kruppel-related gene 1) has been cloned from a human fetal brain cDNA library with 2612 nt. It is indicated that **HKR1** contains 10 highly conserved C2H2-type zinc finger motifs at the C-terminus and 2 KRAB (Kruppel-assocd. box) domains at the N-terminus.
It has been found that the expression of **HKR1** is higher in neuroglioma than those in normal brain tissue with cDNA microarray and in situ hybridization. The results suggest **HKR 1** might be a putative cancer-assocd. gene.

L4 ANSWER 9 OF 13 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 1995:453950 CAPLUS
DOCUMENT NUMBER: 122:260593
TITLE: Hansenura mrakii killer toxin-resistant gene of yeast and its function
AUTHOR(S): Nakajima, Tasuku
CORPORATE SOURCE: Fac. Agric., Tohoku Univ., Sendai, 981, Japan
SOURCE: Kagaku to Seibutsu (1995), 33(3), 146-8
CODEN: KASEAA; ISSN: 0453-073X
DOCUMENT TYPE: Journal; General Review
LANGUAGE: Japanese
AB A review, with 6 refs. of the structure of gene **HKR1**, involved in resistance to H. mrakii killer toxin (HM-1), homol. to MSB2 as a multicopy suppressor gene of yeast cdc24 mutant, and putative function of **HKR1** protein.

L4 ANSWER 10 OF 13 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 1988:523584 CAPLUS
DOCUMENT NUMBER: 109:123584
TITLE: The GLI-Kruppel family of human genes
AUTHOR(S): Ruppert, John M.; Kinzler, Kenneth W.; Wong, Albert J.; Bigner, Sandra H.; Kao, Fa Ten; Law, Martha L.; Seuanez, Hector N.; O'Brien, Stephen J.; Vogelstein, Bert
CORPORATE SOURCE: Sch. Med., Johns Hopkins Univ., Baltimore, MD, 21231, USA
SOURCE: Mol. Cell. Biol. (1988), 8(8), 3104-13

CODEN: MCEBD4; ISSN: 0270-7306
DOCUMENT TYPE: Journal
LANGUAGE: English
AB Previous characterization of GLI, a gene found to be amplified and expressed in a subset of human brain tumors, revealed the presence of five tandem zinc fingers related to those of Krueppel (Kr), a Drosophila segmentation gene of the gap class. The GLI cDNA was used as a mol. probe to isolate related sequences from the human genome. Partial characterization of six related loci, including sequence detn., expression studies, and chromosome localization, revealed that each locus could encode a sep. finger protein. The predicted proteins all had similar H-C links, i.e., a conserved stretch of 9 amino acids connecting the C-terminal histidine of one finger to the N-terminal cysteine of the next. On the basis of amino acid sequence and intron-exon organization, the genes could be placed into one of two subgroups: the GLI subgroup (with the consensus finger amino acid sequence [Y/F]XCX3GCX3[F/Y]X5LX2HX3-4H[T/S]GEKP) or the Kr subgroup (with the consensus finger amino acid sequence [Y/F]XCX2CX3FX5LX2HXRXHTGEKP). Unlike GLI or Kr, most of the newly isolated genes were expressed in many adult tissues. The predicted proteins probably control the expression of other genes and, by analogy with Kr and GLI, may be important in human development, tissue-specific differentiation, or neoplasia.

L4 ANSWER 11 OF 13 LIFESCI COPYRIGHT 2002 CSA
ACCESSION NUMBER: 2001:65516 LIFESCI
TITLE: Genetic assignment of the GLI gene to porcine chromosome 5
AUTHOR: Nielsen, V.; Thomsen, B.
CORPORATE SOURCE: Department of Animal Breeding and Genetics, Danish Institute of Agricultural Sciences, P.O. Box 50, DK-8830 Tjele, Denmark
SOURCE: Animal Genetics [Anim. Genet.], (20001200) vol. 31, no. 6, pp. 409-410.
ISSN: 0268-9146.
DOCUMENT TYPE: Journal
FILE SEGMENT: G
LANGUAGE: English
SUMMARY LANGUAGE: English
AB A 1.55-kbp fragment of the human GLI gene (**glioma-associated oncogene** homologue) was excised with PstI from pKK36PI and used as a probe after radiolabelling.

L4 ANSWER 12 OF 13 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
ACCESSION NUMBER: 1999:345044 BIOSIS
DOCUMENT NUMBER: PREV199900345044
TITLE: Analysis of mRNA levels of Saccharomyces cerevisiae genes involved in cell wall biosynthesis during spheroplast regeneration.
AUTHOR(S): Braley, R. (1); Chaffin, W. L. (1)
CORPORATE SOURCE: (1) Texas Tech University Health Science Center, Lubbock, TX USA
SOURCE: Abstracts of the General Meeting of the American Society for Microbiology, (1999) Vol. 99, pp. 307.
Meeting Info.: 99th General Meeting of the American Society for Microbiology Chicago, Illinois, USA May 30-June 3, 1999
American Society for Microbiology
. ISSN: 1060-2011.
DOCUMENT TYPE: Conference
LANGUAGE: English

L4 ANSWER 13 OF 13 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.

ACCESSION NUMBER: 1997:384872 BIOSIS
DOCUMENT NUMBER: PREV199799684075
TITLE: Effect of irrigation conditions on plant water relations, radiation characteristics and grain yield of rice (Oryza sativa L.) cultivars.
AUTHOR(S): Singh, Harbir; Singh, Tej; Singh, V. P.; Tonk, D. S.; Singh, K. P.; Singh, B. R.
CORPORATE SOURCE: Dep. Agronomy, CCS Haryana Agric. Univ., Hisar 125 004 India
SOURCE: Crop Research (Hisar), (1997) Vol. 13, No. 3, pp. 559-563.
ISSN: 0970-4884.
DOCUMENT TYPE: Article
LANGUAGE: English
AB In a field trial on sandy loam soil at Hisar, five rice varieties (Jaya, HKR 101, Pusa 2-21, PAL 579 and **HKR-1**) were tested under two irrigation variants viz., continuous submergence (5 +- 2 cm) and alternate wetting and drying (hair cracking). Treatments were evaluated in terms of changes in leaf water potential, stomatal conductance, radiation characteristics and grain and straw yield. Continuous submergence maintained higher leaf water potential, stomatal conductance, absorption coefficient, grain and straw yield than alternate wetting and drying irrigation schedule. Among the rice varieties, Jaya and HKR-101 being at par recorded significantly higher grain and straw yield as compared to other varieties. Leaf water potential and absorption coefficient showed significantly linear and positive relationship with grain yield, whereas the transmission coefficient showed significant but negative association with grain yield. Thus, the mid day leaf water potential, stomatal conductance and photosynthetically active radiation can be used for studying the effect of irrigation conditions in transplanted rice in north India.

=>

=>

=> s Hansenula mrakii killer toxin () resistant gene() 1

L5 5 HANSENULA MRAKII KILLER TOXIN (W) RESISTANT GENE(W) 1

=> s l5 and (antisens? or triplex or ribozym?)

L6 0 L5 AND (ANTISENS? OR TRIPLEX OR RIBOZYM?)

=> s human kruppel related gene 1

L7 1 HUMAN KRUPPEL RELATED GENE 1

=> d l7 ibib abs tot

L7 ANSWER 1 OF 1 CAPLUS COPYRIGHT 2002 ACS
ACCESSION NUMBER: 2001:93856 CAPLUS
DOCUMENT NUMBER: 135:238195
TITLE: Expression analysis of zinc finger protein HKR1 in neuroglioma
AUTHOR(S): Xia, Fang; Qi, Zhen-yu; Dai, Jian-liang; Gu, Shao-hua; Ma, Liang-xiao; Xie, Yi; Mao, Yu-min
CORPORATE SOURCE: Institute of Genetics, School of Life Sciences, Fudan University, Shanghai, 200433, Peop. Rep. China
SOURCE: Fudan Xuebao, Ziran Kexueban (2000), 39(6), 692-694

CODEN: FHPTAY; ISSN: 0427-7104
PUBLISHER: Fu[redacted] Daxue Chubanshe
DOCUMENT TYPE: Jo[redacted]al
LANGUAGE: Chinese
AB The cDNA of HKR1 (**Human kruppel-related gene 1**) has been cloned from a human fetal brain cDNA library with 2612 nt. It is indicated that HKR1 contains 10 highly conserved C2H2-type zinc finger motifs at the C-terminus and 2 KRAB (Kruppel-assocd. box) domains at the N-terminus. It has been found that the expression of HKR1 is higher in neuroglioma than those in normal brain tissue with cDNA microarray and in situ hybridization. The results suggest HKR 1 might be a putative cancer-assocd. gene.

=> s human kruppel related gene() 1

L8 1 HUMAN KRUPPEL RELATED GENE(W) 1

=> s human kruppel related 1

L9 0 HUMAN KRUPPEL RELATED 1

STIC-ILL

QH506.M6

From: Lacourciere, Karen
Sent: Friday, May 10, 2002 3:43 PM
To: STIC-ILL
Subject: ill order

395758

Please provide the following references for use in examining 09/898,556

Mol. Cell. Biol. (1988), 8(8), 3104-13

2850480

Kagaku to Seibutsu (1995), 33(3), 146-8

Fudan Xuebao, Ziran Kexueban (2000), 39(6), 692-694

JOURNAL OF BACTERIOLOGY, (1994 Mar) 176 (5) 1488-99.

JOURNAL OF BACTERIOLOGY, (1996 Jan) 178 (2) 477-83.

GENE, (1998 Nov 5) 222 (1) 61-7.

MOLECULAR AND GENERAL GENETICS, (1999 Mar) 261 (2) 275-80.

ANIMAL GENETICS, (2001 Dec) 32 (6) 375-9.

Thank-you!

6910164

Karen A. Lacourciere Ph.D.
CM1 11D09 GAU 1635
(703) 308-7523

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

MOLECULAR AND CELLULAR BIOLOGY, Aug. 1988, p. 3104–3113
0270-7306/88/083104-10$02.00/0

Vol. 8, No. 8

The *GLI*-Kruppel Family of Human Genes

JOHN M. RUPPERT,[1] KENNETH W. KINZLER,[1,2] ALBERT J. WONG,[1] SANDRA H. BIGNER,[3] FA-TEN KAO,[4] MARTHA L. LAW,[4] HECTOR N. SEUANEZ,[5] STEPHEN J. O'BRIEN,[5] AND BERT VOGELSTEIN[1]*

The Oncology Center[1] and Department of Pharmacology and Molecular Sciences,[2] Johns Hopkins University School of Medicine, Baltimore, Maryland 21231; Department of Pathology, Duke University Medical Center, Durham, North Carolina 27710[3]; Eleanor Roosevelt Institute for Cancer Research and School of Medicine, University of Colorado Health Sciences Center, Denver, Colorado 80262[4]; and Laboratory of Viral Carcinogenesis, National Cancer Institute, Frederick, Maryland 21701[5]

Received 24 February 1988/Accepted 9 May 1988

Previous characterization of *GLI*, a gene found to be amplified and expressed in a subset of human brain tumors, revealed the presence of five tandem zinc fingers related to those of Krüppel (*Kr*), a *Drosophila* segmentation gene of the gap class. We have used the *GLI* cDNA as a molecular probe to isolate related sequences from the human genome. Partial characterization of six related loci, including sequence determination, expression studies, and chromosome localization, revealed that each locus could encode a separate finger protein. The predicted proteins all had similar H-C links, i.e., a conserved stretch of 9 amino acids connecting the C-terminal histidine of one finger to the N-terminal cysteine of the next. On the basis of amino acid sequence and intron-exon organization, the genes could be placed into one of two subgroups: the *GLI* subgroup (with the consensus finger amino acid sequence [Y/F]XCX$_3$GCX$_3$[F/Y]X$_5$LX$_2$HX$_{3-4}$H[T/S]GEKP) or the *Kr* subgroup (with the consensus finger amino acid sequence [Y/F]XCX$_2$CX$_3$FX$_5$LX$_2$HXRXHTGEKP). Unlike *GLI* or *Kr*, most of the newly isolated genes were expressed in many adult tissues. The predicted proteins probably control the expression of other genes and, by analogy with *Kr* and *GLI*, may be important in human development, tissue-specific differentiation, or neoplasia.

One of the principal objectives of molecular biology is to gain an understanding of mechanisms resulting in specific temporal and spacial patterns of gene expression. Among the proteins important in this regard are those containing distinct DNA-binding regions. In *Drosophila melanogaster*, two classes of such proteins have been identified, containing either homeo box regions or zinc fingers in their putative DNA-binding domains. Homeo box regions were first detected in the antennapedia and fushi tarazu genes and have subsequently been identified in other *Drosophila* developmental genes (19). The homeo box elements are conserved in genes of a diverse array of species, and recent experiments have indicated a role for such proteins in mammalian embryonic development (for a review, see reference 20).

Zinc fingers were first identified in the *Xenopus* transcription factor TFIIIA (6, 37). Such fingers have been proposed to bind specific nucleic acid sequences while tetrahedrally coordinating a metal ion (zinc) via conserved cysteine and histidine residues (23, 37). Zinc fingers have been found in many regulatory genes (2, 16, 32, 58, 62). A family of zinc finger genes related to Krüppel (*Kr*) is particularly relevant to the present work. The *Drosophila* gene *Kr* is a member of the gap class of segmentation genes, and thoracic and anterior abdominal segments fail to form in *Kr* mutant embryos (44). *Kr* encodes a chromatin-associated phosphoprotein (41) which contains five zinc fingers with the consensus sequence (Y/F)XCX$_2$CX$_3$FX$_5$LX$_2$HX$_3$HTGEKP, in which X can be any amino acid (46). These consensus features, including the H-C link (the amino acid sequence HTGEKP(Y/F)XC connecting the histidine of one finger to the cysteine of the next), define the Krüppel family of zinc finger genes (49). Conservation of the contiguous stretch of nucleotides encoding the H-C link has allowed cloning of Krüppel-related genes from *D. melanogaster*, mouse, and frog by hybridization with Krüppel cDNA at low stringency (10, 47, 49). Each of these Krüppel family members has been shown to be expressed in embryonic cells, suggesting a role for them in normal development.

Our interest in this family of genes was sparked by the identification of the *GLI* gene as a member of the Krüppel family (31). The *GLI* gene was discovered by virtue of its amplification in a subset of human brain tumors (30). Sequencing of *GLI* cDNA clones revealed the presence of five tandem fingers connected by H-C links similar to those of Krüppel. The fact that a structural motif proposed to mediate sequence-specific nucleic acid binding is found both in *Drosophila* developmental genes and in a gene implicated in human neoplasia suggested that other genes of this class might prove important in normal or disease states. Indeed, other genes important in neoplasia, such as *N-NYC* (33, 50), *L-MYC* (38), *HER-2* (12, 29), and *N-RAS* (54), were identified partly through their homology to previously identified oncogenes. Similarly, genes of potential importance in development have been identified through sequence similarity to known developmental genes (e.g., the *HOX* family of genes in mammals; 11, 34, 35). We have therefore used a *GLI* cDNA fragment which encodes the finger region to isolate related human sequences. Six distinct loci were identified in this manner and shown to be present on five different chromosomes. Partial sequencing revealed that each had open reading frames capable of encoding fingers with H-C links. Unlike Krüppel family genes previously identified in other species, most of these sequences were expressed in several adult tissues. Further study of these genes may provide insights into transcriptional mechanisms, normal human development, and/or neoplasia.

* Corresponding author.

MATERIALS AND METHODS

Library construction. Genomic DNA was purified from mouse xenografts of the glioblastoma multiforme cell line D320MG (25a) as described previously (61). After partial *Mbo*I digestion, DNA was size fractionated by sucrose density gradient ultracentrifugation. The fractions containing 17- to 24-kilobase fragments were cloned into the *Bam*HI site of Lambda Fix (Stratagene) after partial fill in of *Mbo*I ends according to the instructions of the manufacturer. The ligation product was packaged with lambda phage extracts (Stratagene) and used to infect *Escherichia coli* C600 cells. DNA from the resulting plaques was lifted with Colony Plaque Screen nylon membrane (Dupont, NEN Research Products) and bound by treatment with 0.4 M NaOH for 15 min (45). The filters were hybridized to a *GLI* finger probe (pGLIMBD) containing a 395-base-pair fragment of *GLI* cDNA spanning fingers 2 to 5 and containing nucleotides 873 to 1267 (31). Three rounds of plaque selection via hybridization and subsequent replating were used before purified DNA from individual plaques was analyzed.

DNA hybridization. After digestion with *Eco*RI or *Hin*dIII, genomic or phage DNA fragments were separated by electrophoresis through 1% agarose gels and transferred to nylon as described (45). DNA was labeled with ^{32}P by oligo labeling (17) and hybridized as described (61), except that nonfat dried milk was used (0.5%) (26), and 10% polyethylene glycol was included. For reduced-stringency experiments, Southern blots or plaque lifts were hybridized at 55°C and washed at 55 to 60°C in 0.3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.3% sodium dodecyl sulfate (SDS) for 1 h. For normal stringency, the same wash solution was used but at 65°C for 1 h.

Plasmid subcloning. For isolation of subclones from recombinant bacteriophage, *Eco*RI, *Hin*dIII, or *Eco*RI-*Sal*I fragments which hybridized to pGLIMBD were separated from other fragments by electrophoresis and eluted from the gel (60). (*Sal*I sites are adjacent to the *Bam*HI cloning sites in Lambda Fix). Fragments were subcloned in alkaline phosphatase-treated Bluescript M13 + KS vector (Stratagene). For isolation of finger-containing sequences in smaller fragments for sequencing, these plasmid subclones were digested with several restriction endonucleases which had 4-bp recognition sites. The fragments were separated by electrophoresis in 1.5% agarose gels and studied by Southern blot analysis by using the pGLIMBD probe. Hybridizing fragments were eluted from agarose gels, and the ends were filled in with the Klenow fragment of *Pol*I. These blunt-ended fragments were ligated into the *Eco*RV site of Bluescript KS. Resulting clones were digested with *Eco*RI and *Hin*dIII, and the inserts were recloned into the same sites of Bluescript SK for sequencing in the opposite direction.

Thus, for each of the six loci indicated in Fig. 1, three sets of clones were generated: the original phage clone, a plasmid subclone with a large insert containing the finger region (Fig. 1), and small subclones of these plasmids which contained the pGLIMBD hybridizing regions and which were used for sequencing (Table 1). Two phages contained sequences that hybridized to non-zinc finger regions of the *GLI* cDNA probe (see text). Subcloning and sequencing of these regions were done in the same way as described above for the finger regions. The large subclone of GLI2 which hybridized to the upstream probe of *GLI* contained the 1.0-kilobase *Eco*RI fragment located 7.6 kilobases to the left of pGLI2RR (Fig. 1).

Sequencing. Sequencing was performed by the chain termination method with modified T7 polymerase (53). Single-strand templates were obtained from subclones in Bluescript by using f1 helper phage R408 (48). Most sequences were obtained by using data from both strands. The labeling and electrophoresis conditions we employed precluded sequencing of the terminal 10 to 15 bases at the end of each plasmid insert.

Ribonuclease protection. Total RNA was isolated by the acid-guanidium extraction method described by Chomczynski and Sacchi (9). ^{32}P-labeled RNA transcripts were generated in vitro from the sequencing subclones described above by using T3 or T7 RNA polymerase. Ribonuclease protection was performed as described (63) with the following modifications: hybridizations were performed in a final volume of 10 μl; only RNase A at 12.5 μg per ml was used; and the RNase A and proteinase K digestions were performed at room temperature for 30 min. Tera-1 cells (18) were obtained from the American Type Culture Collection. NTera-2 was generously provided by P. Andrews (1).

Chromosome localization. Southern blot analysis of human-rodent somatic cell hybrid DNA (8, 28, 39, 40) was performed by using inserts from the sequencing subclones described above.

RESULTS

Isolation of *GLI*-related sequences. To identify *GLI*-related sequences, a fragment of *GLI* cDNA encoding fingers two to five was subcloned (pGLIMBD) as described in Materials and Methods. Reduced stringency hybridization with this probe to Southern blots of human DNA revealed several bands not seen at normal stringency (not shown). To clone these sequences, 800,000 recombinant bacteriophage (4.5 genome equivalents) from a human genomic library were screened by using the same reduced stringency conditions. Fourteen phages were identified through this screen. Analysis of phage DNA revealed seven unique restriction maps. One set of clones represented the *GLI* locus, and the others represented six new loci. Comparison of the sizes of hybridizing fragments in the phage clones with those seen in genomic Southern blots revealed that most of the sequences detected in the Southern blots had been cloned.

***GLI*-related sequences encoded fingers similar to *GLI* or *Kr*.** Representative phages from each of the six loci defined by restriction mapping were chosen for further study (Fig. 1). For each of the loci, regions of the phage which hybridized to pGLIMBD were subcloned in plasmid vectors. Sequencing of these subclones revealed open reading frames encoding fingers with H-C links for each of the six loci (Fig. 2). No in-frame stop codons were found within the fingers. Intron-exon junctions were predicted adjacent to the finger regions on the basis of consensus splice sequences (51).

Analysis of the predicted amino acid sequences showed that the clones could be placed into one of two subgroups. Two of the six loci (*GLI2* and *GLI3*) encoded fingers that were very similar to those of *GLI* and *MGLI* (the mouse homolog of *GLI*). *GLI2* and *GLI3* had 89% and 84% of their residues in common with *GLI* in the finger regions, respectively (Fig. 3A). Over the 50-amino-acid region for which *GLI*, *GLI2*, and *GLI3* sequences were all available (i.e., from the middle of finger 1 to the amino terminus of finger 3), *GLI2* and *GLI3* were more similar to each other (92% amino acid identity) than either was to *GLI* (84% amino acid identity for each).

Alignment of *GLI2*, *GLI3*, and *MGLI* genomic sequences with the *GLI* cDNA sequence and identification of consen-

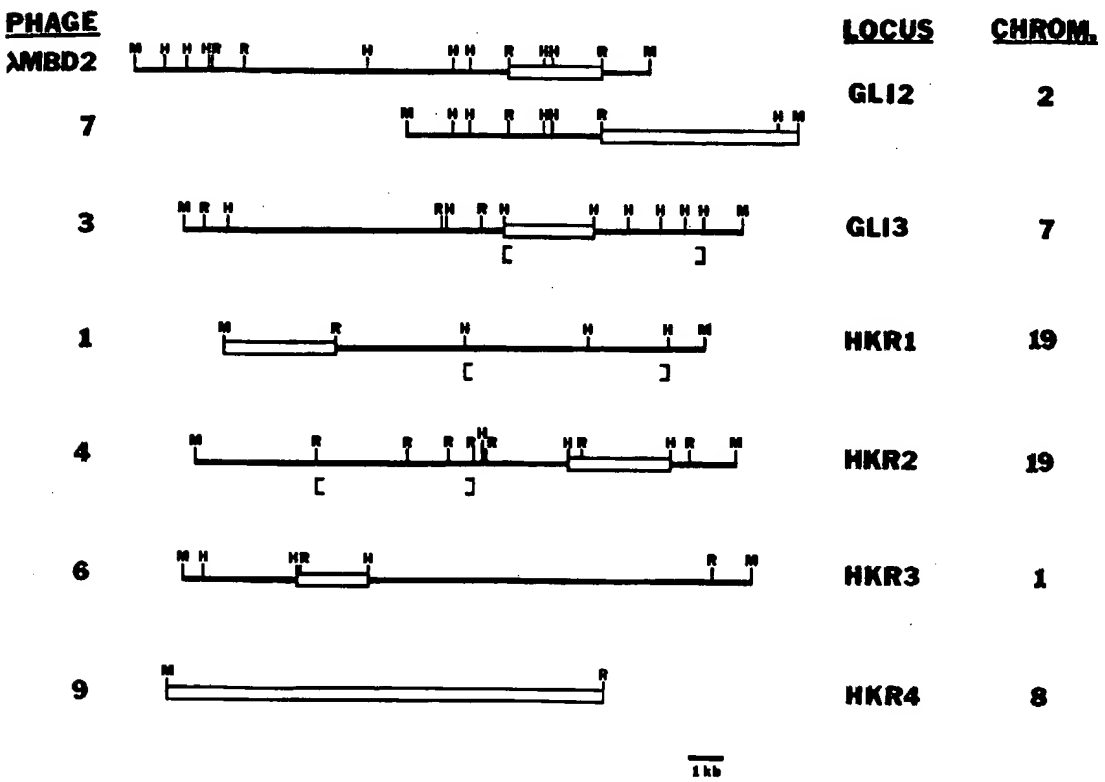

FIG. 1. *GLI*-related loci. Reduced-stringency hybridization of pGLIMBD to a λ phage library allowed isolation of clones containing related sequences. Representative phage for each of the six new loci identified are shown. *Eco*RI (R) and *Hin*dIII (H) recognition sites within the phages are illustrated. M indicates the *Mbo*I site at the junction between human and phage sequences. Brackets below the maps indicate restriction fragments not ordered with respect to one another. Subcloned regions which hybridize to pGLIMBD are shown as open boxes (for plasmid names see Table 1). Hybridization of plasmid subclones to DNA from human-rodent hybrid panels was used to determine chromosome localizations of the six different loci (shown on the right).

sus splice donor and acceptor sequences allowed prediction of intron-exon junctions. Each of the nine predicted intron-exon junction sequences examined showed that the positions of predicted splice junctions within the finger region were exactly conserved (Fig. 3A). For both *GLI2* and *GLI3*, the sequences predicted an exon extending from the middle of finger 1 to near the beginning of finger 3. For *GLI2*, the sequence predicted another exon extending from upstream of the middle of finger 3 (where the sequence was truncated during cloning) to the C-terminus of finger 4. The conservation of intron-exon junctions and the conservation of amino acid sequences within individual fingers supported the hypothesis of a common evolutionary origin for this subgroup of genes (the *GLI* subgroup).

The four other finger-related clones demonstrated amino acid and nucleotide similarity to *GLI* only in the H-C link and in the other consensus residues (i.e., C-C-F-L-H-H) found commonly in many finger proteins. The H-C link

TABLE 1. Loci, phages, and clones used in this study

Locus	Phage	Large plasmid subclone[a]	Sequencing plasmid subclone
GLI2	λMBD2	pGLI2RR	pGLI2RR-*Bst*NI-600
GLI2	λMBD7	pGLI2RS	pGLI2RS-*Taq*I-480[b]
GLI3	λMBD3	pGLI3HH	pGLI3HH-*Hae*III-640[b]
HKR1	λMBD1	pHKR1RS	pHKR1RS-*Rsa*I-680[b]
HKR2	λMBD4	pHKR2HH	pHKR2HH-*Hin*fI-325[b]
HKR3	λMBD6	pHKR3HH	pHKR3HH-*Msp*I-490[b]
HKR4	λMBD9	pHKR4RS	pHKR4RS-*Bst*NI-400
HKR4	λMBD9	pHKR4RS	pHKR4RS-*Bst*NI-450[b]
GLI2	λMBD2	pGLI2RR-2	pGLI2RR-2-*Hae*III-440[c]
HKR3	λMBD6	pHKR3HH	pHKR3HH-HPX-550[c]

[a] The plasmid subclone names include the genomic restriction fragment used as insert (H, *Hin*dIII; R, *Eco*RI; S, *Sal*I; P, *Pst*I; X, *Xba*I) and, in the case of the small subclones used for sequencing, the size of the insert. Thus, the plasmid pGLI2RR-*Bst*NI-600 refers to a plasmid subclone of pGLI2 RR (boxed in Fig. 1) containing a 600-bp *Bst*NI insert.

[b] Subclone used for RNase protection, chromosome localization, and cross-species hybridization studies.

[c] Subclone isolated by hybridization with non-zinc finger regions of *GLI* cDNA (see Materials and Methods).

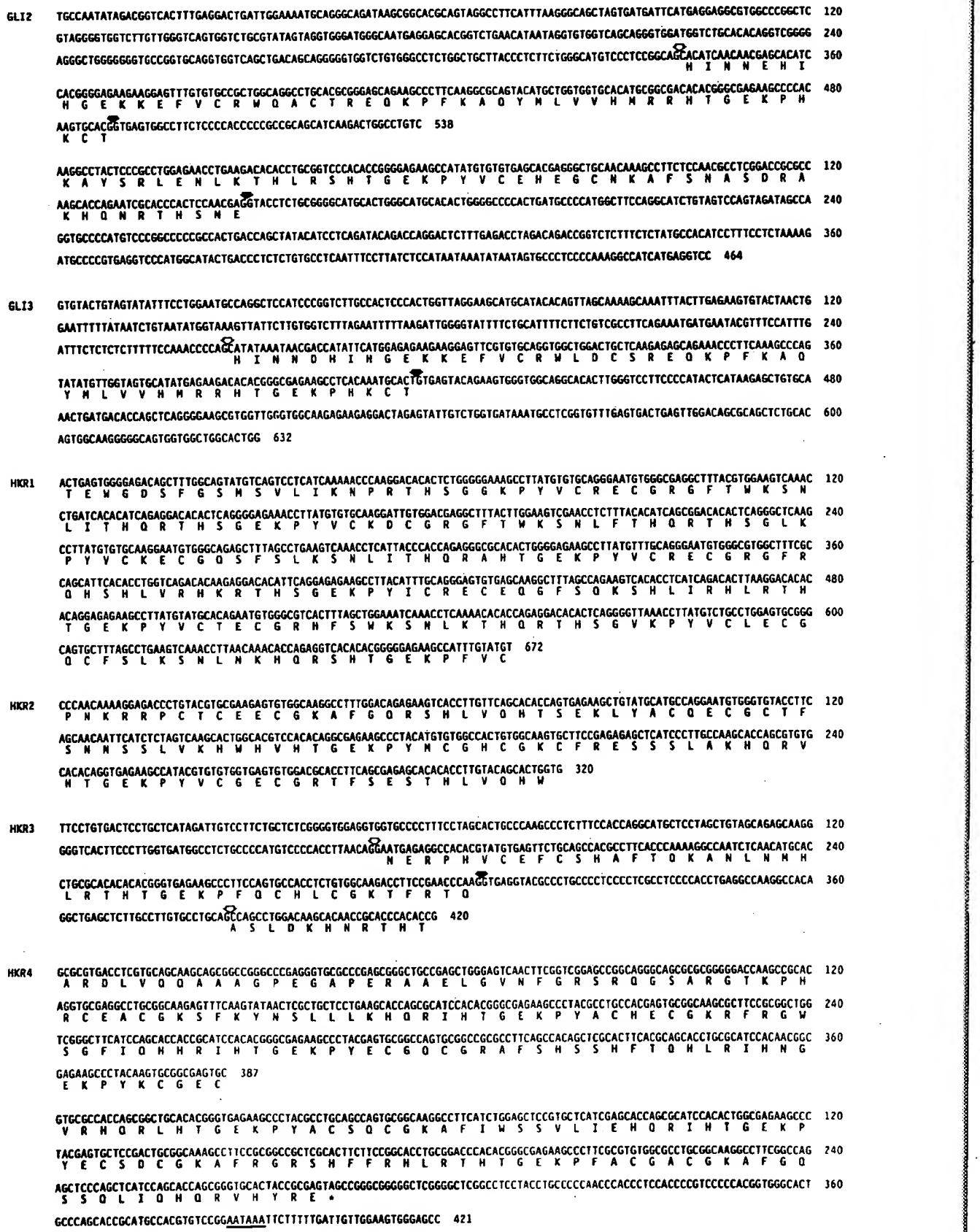

FIG. 2. Sequence analysis of *GLI*-related loci. Sequences of *GLI2*, *GLI3*, *HKR1*, *HKR2*, *HKR3*, and *HKR4* are shown. Sequencing was performed as described in Materials and Methods. Splice consensus sequences (51) were present adjacent to regions of *GLI* cDNA similarity. Closed arrows represent predicted splice donor sites, and open arrows show predicted splice acceptor sites. The sequences obtained for *HKR1* and *HKR2* each had one continuous open reading frame encoding fingers. The *GLI2* and *HKR3* sequences each predicted two different exons. Two *Bst*NI fragments from *HKR4* contained finger-hybridizing sequences; it is not known whether these two *Bst*NI fragments were contiguous in the genome. The presence of an in-frame stop codon (asterisk) in the larger fragment of *HKR4*, the results of ribonuclease protection (Fig. 4), and the presence of a possible polyadenylation signal (underlined) downstream of the stop codon suggested that these sequences lie at the C-terminus of the *HKR4* protein.

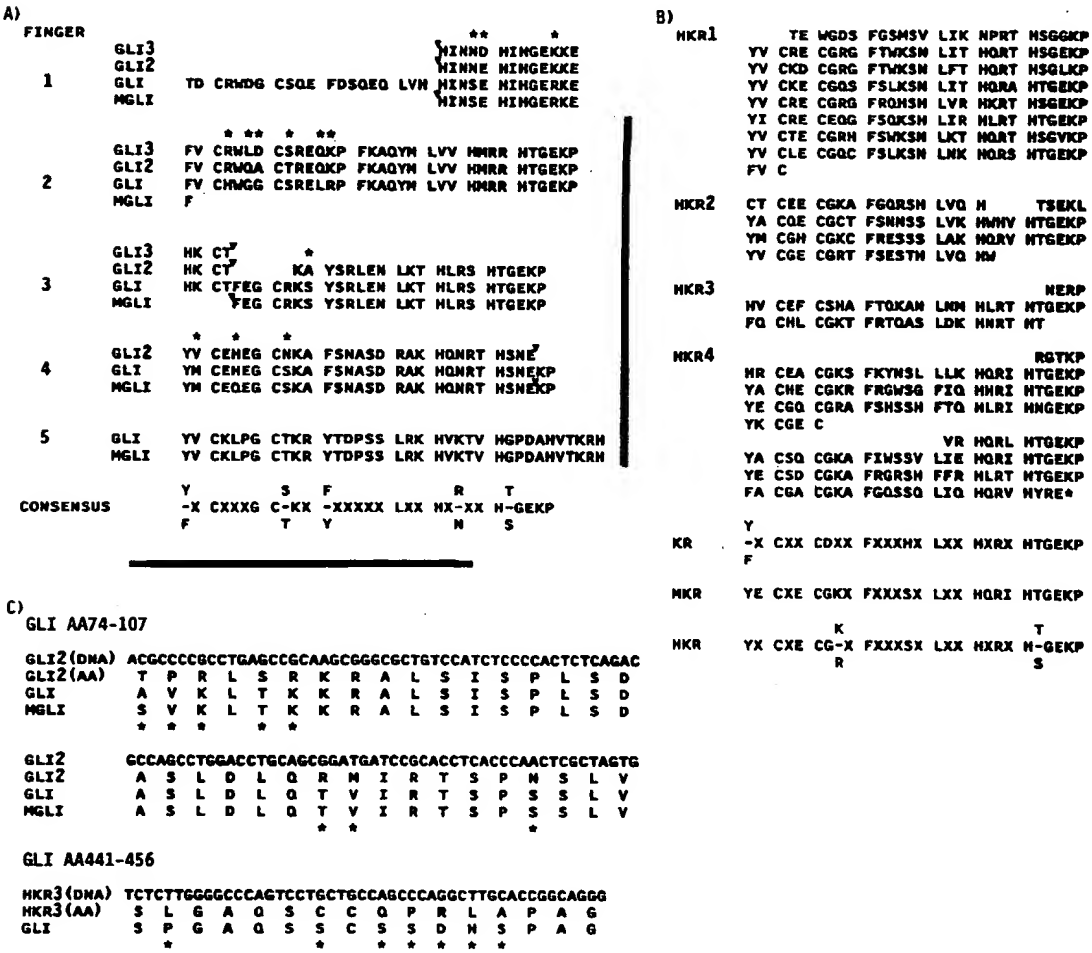

FIG. 3. (A) *GLI* family finger structure. Predicted amino acid sequences obtained from *GLI2*, *GLI3*, and *MGLI* genomic DNA sequences were aligned with *GLI* amino acids 235 to 397. The asterisks mark positions where amino acids varied. Arrowheads show positions of predicted intron-exon junctions (see text and Fig. 2). A consensus sequence common to the majority of *GLI* fingers is shown at the bottom. X indicates any amino acid. (B) Krüppel family structure. Predicted amino acid sequences obtained from *HKR1* to *4* genomic DNA sequences are illustrated, with alignment of fingers to demonstrate the consensus features typical of Krüppel-related genes. The consensus sequences of the fingers for the *HKR*, mouse Krüppel (*MKR*, [10]) and Krüppel (46) genes are shown at the bottom; the asterisk indicates a translation stop codon. (C) Similarity to *GLI* in nonfinger regions. Phage from each of the six loci depicted in Fig. 1 were hybridized with *GLI* cDNA probes corresponding to regions upstream or downstream of the fingers (see text). Subclones containing the hybridizing regions from *GLI2* and *HKR3* were sequenced. These sequences were aligned with the GLI cDNA and the predicted amino acids were compared. The asterisks indicate positions of amino acid variation.

accounts for the only contiguous stretch of *GLI* similarity and thus was responsible for the hybridization to these genes. Inspection of the predicted amino acid sequences of the four clones (Fig. 3B) showed that they were more similar to previously described *Kr* family genes than to the *GLI* genes described above. The four loci were therefore named *HKR1* to *4* (for human Krüppel-related genes). Some *HKR* fingers had up to 75% identity with those of mouse (*MKR1*, *MKR2*, [10]) or *Xenopus* (*Xfin* [47]) Krüppel-related genes. For example, one of the fingers of *HKR4* shared 78% identity with one finger of *MKR2* and 75% identity with the finger 34 of *Xfin*. However, this strong similarity was only observed in isolated fingers, and none of the four *HKR* genes appeared to represent the human homolog of a previously described zinc finger gene.

Spacing of the first two cysteines within *HKR* gene fingers (CX_2C) differed from the *GLI* gene fingers (CX_4C). In addition, inspection of the finger sequences revealed specific conserved amino acids that distinguished the *HKR* subgroup from the *GLI* subgroup (Fig. 3A and B). Those amino acids that were common to *HKR* fingers were also common to those of the mouse Krüppel genes (Fig. 3B). Another difference between the *GLI* and *HKR* genes was that *HKR* had several fingers encoded by one exon, while in *GLI* exons generally encoded only one complete finger (Fig. 2). The mouse Krüppel family genes also encode multiple fingers in individual exons (10).

For *HKR4*, an in-frame stop codon occurred at the carboxy terminus of a predicted finger, followed by a possible polyadenylation signal (AATAAA) 103 base pairs downstream (Fig. 3B). Location of a finger region at the C-terminus has also been observed in the *Drosophila* finger protein gene *snail* (5).

Similarity in nonfinger regions. It was of interest to determine whether the six loci described above contained regions of similarity to *GLI* other than those in the zinc finger

domain. Towards this end, phage clones representing each of the six loci were hybridized to *GLI* cDNA probes containing coding sequences upstream (nucleotides 118 to 873) or downstream (nucleotides 1255 to 2426) of the fingers. *GLI2* was found to hybridize to the upstream probe, while *HKR3* hybridized to the downstream probe. Genomic sequences from the hybridizing regions of *GLI2* and *HKR3* were subcloned and sequenced, and the predicted amino acid sequences were compared to *GLI* (Fig. 3C). *GLI2* and *GLI* showed 76% identity over a 34-amino-acid region upstream of the *GLI* fingers (amino acids 74 to 107 of *GLI*). Over the same region, *MGLI* and *GLI* were identical at all but the first position. *HKR3* and *GLI* shared 56% of residues over a 16-amino-acid region downstream of the *GLI* zinc fingers (amino acids 441 to 456 of *GLI*).

Expression of finger-encoding genes. To assess expression, subclones containing finger regions from each of the six loci described in Fig. 1 were used to generate synthetic antisense RNA transcripts. RNA from normal human tissues, a glioblastoma tumor (D245MG [4]), and two embryonal carcinoma cell lines (Tera-1 [18] and NTera-2 [1]) were then tested for their abilities to protect the in vitro transcripts from RNase A digestion. The glioblastoma and embryonal carcinoma cell lines were chosen because previous studies had indicated that *GLI* and other Krüppel-related genes were expressed in analogous cells (10, 31).

Expression was demonstrated for all loci except *HKR2* (Fig. 4). In each case, the length of the protected RNA transcript corresponded to that expected from sequence data (Fig. 2). The levels of expression in different cell types varied widely among the six loci. *GLI2* was found to be expressed in all normal tissues except for placenta, with highest levels expressed in testes, myometrium, and kidney. In contrast, *GLI3* was found to be expressed in all normal tissues except for kidney and brain, with highest levels in myometrium and lung. *HKR1* and *HKR4* were found to be expressed at detectable levels in all normal tissues studied, while *HKR3* was found in all except placenta. For each of the expressed loci, levels of expression were considerably higher in the glioblastoma multiforme or embryonal carcinoma cell lines than in any normal tissue.

Similar sequences in other species. The data on RNA expression showed that five of the six loci contained genes which were expressed in normal adult tissues. Further evidence for the potential importance of these genes was provided by the observation that sequences related to each of the genes were evolutionarily conserved. Human, mouse, rat, chicken, frog, fly, and yeast DNAs were digested with *Eco*RI or *Hin*dIII, and Southern blots were prepared. At reduced stringencies (see Materials and Methods), probes containing finger-encoding regions from each of the six loci identified one or a few prominent restriction fragments in various other species (Fig. 5 and data not shown). For example, *HKR1* detected related fragments in mouse and rat (Fig. 5A), whereas *GLI3* detected fragments in every species tested (Fig. 5B). *HKR3* identified several fragments in every species except for yeast and frog. Comparison of the sizes of the *Eco*RI and *Hin*dIII fragments identified by each of the *HKR* loci revealed that each hybridized to a different set of restriction fragments. Therefore, the H-C link, which was present in all six loci, was not responsible for the cross-species hybridization patterns. Rather, the patterns indicated that sequences within each of the identified loci were evolutionarily conserved separately. For two of the six probes (*HKR3* and *HKR4*), further reduction of stringency allowed visualization of many additional fragments in all species tested except for yeast (cf. Fig. 5C and 5D).

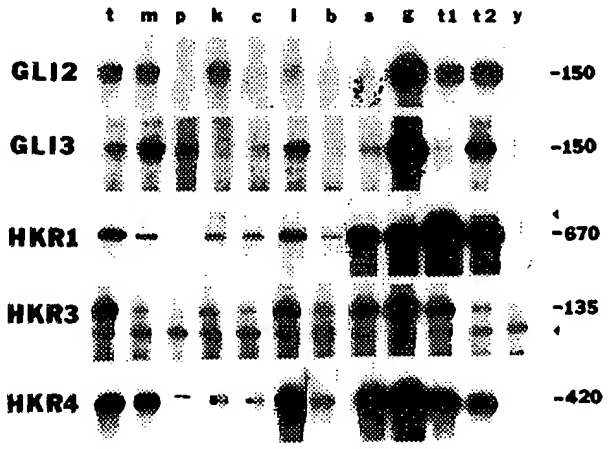

FIG. 4. Expression of *GLI-Kr*. Expression was assessed by protection of labeled antisense RNA from ribonuclease digestion. Labeled transcripts of the plasmid subclones corresponding to each of the six loci depicted in Fig. 1 were hybridized with 20 μg of total RNA from the sources listed below. After digestion with ribonuclease A, RNA was separated by electrophoresis on denaturing polyacrylamide gels and exposed to autoradiographic film. The plasmid subclones used for protection are described in Materials and Methods. None of the RNA samples analyzed protected *HKR2*. The sizes of the protected transcripts, shown on the right in bases, correspond to those expected from sequence data and support the intron-exon junction predictions (Fig. 2). Doublets can be seen in some lanes with the *GLI2* and *HKR4* probes. It is not known whether these resulted from incomplete RNase protection in vitro or alternative RNA processing in vivo. Arrowheads indicate nonspecific signals present in all lanes, including yeast tRNA lanes. Lanes: t, testes; m, myometrium; p, placenta; k, kidney; c, colon; l, lung; b, brain; s, spleen; g, glioblastoma multiforme D245MG; t1, embryonal carcinoma cell line Tera-1; t2, embryonal carcinoma cell line NTera-2; y, yeast tRNA.

Chromosome localization of the six loci. The data on RNA expression (Fig. 4) and species conservation (Fig. 5) indicated significant differences among the six loci, and were consistent with the hypothesis that each encoded a different gene. Further evidence for this hypothesis was provided by chromosome localization studies. DNA from human-rodent hybrid panels (8, 28, 40) was used in hybridization studies with probes representing each of the six loci. The six loci were found to be localized to five different chromosomes (Fig. 1). These chromosome localizations, based on karyotypic and enzymatic analysis of hybrids, were confirmed by syntenic mapping, wherein probes from each of the chromosomes predicted to contain one of the six genes were rehybridized to the same Southern blots, duplicating the initial patterns. Thus, the segregation of *GLI2* was concordant with *N-MYC* on chromosome 2, *GLI3* with the epidermal growth factor receptor gene on chromosome 7, *HKR1* and 2 with *APO-E* on chromosome 19, *HKR3* with *N-RAS* on chromosome 1, and *HKR4* with *C-MYC* on chromosome 8.

DISCUSSION

We used a *GLI* finger probe to isolate sequences from six loci, each of which encoded zinc fingers with conserved H-C links. By analogy with previously characterized zinc finger

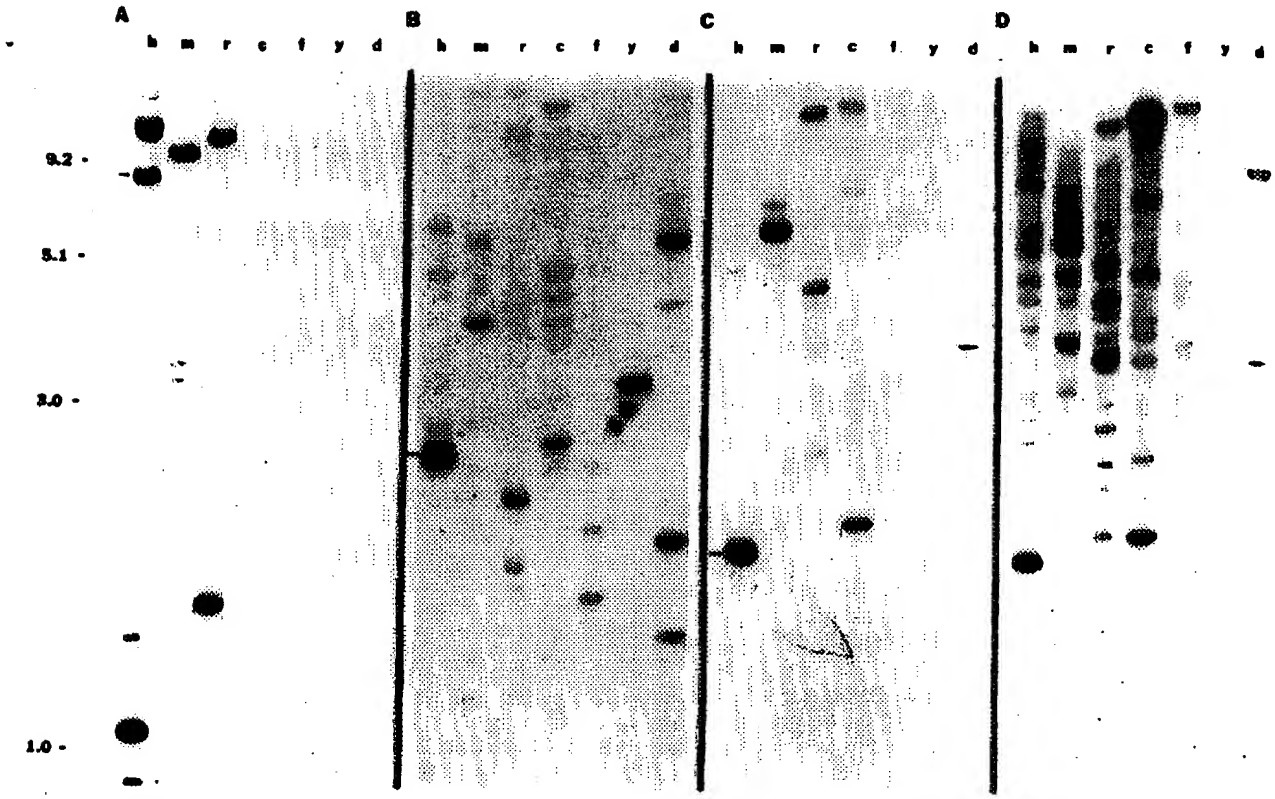

FIG. 5. Evolutionary conservation of finger-encoding sequences. Southern blots containing DNA from several eucaryotes (listed below) were hybridized at reduced stringency to radiolabeled DNA probes prepared from the subclones described in Materials and Methods. Panels A, B, and C show *HKR1*, *GLI3*, and *HKR3* probes hybridized at moderate stringency. Panel D shows hybridization to the *HKR3* probe at lower stringency. Arrows indicate the unique human *Hin*dIII fragments which hybridized at normal stringency (data not shown). Numbers on the left are kilobases. Lanes: h, human (5 μg); m, mouse (5 μg); r, rat (5 μg); c, chicken (5 μg); f, frog (5 μg); y, yeast (1 μg); d, *D. melanogaster* (1 μg).

genes (6, 27, 37), these probably encode DNA- (or RNA) binding proteins which may regulate transcription. At least two of the loci (*HKR3* and *GLI2*) contained sequences related to other parts of the *GLI* cDNA as well, suggesting that the putative proteins encoded by these loci have *GLI* similarity aside from that in the finger region. Five of the six loci were found to be variably expressed in several normal adult tissues as well as in embryonal carcinoma cell lines. The genes were mapped to five different chromosomes, and each was evolutionarily conserved. These studies, therefore, document the basic features of a family of human genes which have in common an H-C link connecting zinc fingers. There are probably additional members of this family besides the seven genes discussed here (*GLI*, *GLI2*, *GLI3*, and *HKR1 -2*, *-3*, and *-4*), since low-stringency hybridization with probes from some of these loci revealed the presence of many restriction fragments (Fig. 5D).

Conservation of DNA-binding protein motifs has allowed isolation of related genes within families in the case of homeo box-containing genes (11, 34, 35) and for two classes of finger proteins, the nuclear receptor family (e.g., estrogen [22] or retinoic acid [21, 43] receptors) and the Krüppel family. The Krüppel family genes have been isolated from several species; some have been isolated by hybridization with Krüppel cDNA (7, 10, 47, 49), and others have been cloned independently (see Table 2). In general, similarity outside of the finger region has not been demonstrated between Krüppel family members (other than the small regions of similarity described in Fig. 3C and in Chavrier et al. [7]), suggesting a significant diversity of function.

Because of the large number of finger-containing genes, it is useful to subclassify them on the basis of common conserved elements (2, 16, 32). One major class of finger proteins, termed C_2H_2 (16), contains several adjacent fingers of the form $(Y/F)XCX_{2-4}CX_3$ $FX_5LX_{2-3}HX_{3-4}HX_5$. This class is distinguished from other classes of finger proteins by virtue of the fact that the putative metal-binding amino acids are always C-C- - -H-H, and phenylalanine and leucine residues are present near the finger midpoints. Three subclasses with these features can be defined (Table 2). The C_2H_2-X_5 subclass genes lack any consensus sequence connecting the zinc fingers. In contrast, the *GLI*-Krüppel gene family members are notable for having a conserved H-C link (HTGEKP(Y/F)XC) present between most fingers (10, 49). Identical H-C links are only occasionally found in C_2H_2-X_5 proteins (e.g., between one pair of the nine fingers of *TFIIIA*). The *GLI*-Krüppel genes can be divided into Krüppel (C_2H_2-*Kr*) or *GLI* (C_2H_2-*GLI*) subclasses, depending on spacing within fingers and other conserved primary sequence features (Fig. 3A and B; Table 2). Interestingly, the *Sp1* gene was recently sequenced and found to have three zinc fingers with connecting H-C links, which is typical of the *GLI*-Krüppel family (27). One finger of *Sp1* fits the *Kr* subgroup consensus, one finger fits the *GLI* subgroup consensus, and one finger fits neither. In contrast, the six genes described in this report were clearly divisible into *GLI* or

TABLE 2. C_2H_2 finger proteins

Subclass	H-C links	Consensus finger sequence	Gene	Source	Reference	Method of isolation
C_2H_2-X_5	Minority of fingers	(Y/F)XCX$_{2-4}$CX$_3$FX$_5$LX$_{2-3}$HX$_{3-4}$HX$_5$	*TFIIIA*	*Xenopus*	6, 37	Independent
			serendipity	*Drosophila*	59	Independent
			hunchback	*Drosophila*	55	independent
			snail	*Drosophila*	5	Independent
			pDP1007	Human	42	Independent
C_2H_2-*KR*	Most fingers	(Y/F)XCX$_2$CX$_3$FX$_5$LX$_2$HXRXHTGEKP	Krüppel	*Drosophila*	46	Independent
			ADR1	Yeast	24	Independent
			KR-H	*Drosophila*	49	*KR*-related
			Xfin	*Xenopus*	47	*KR*-related
			NGFI-A	Rat	36	Independent
			MKR1,2	Mouse	10	*KR*-related
			KROX4,6,8,9,20	Mouse	7	*KR*-related
			EGR-1	Mouse	52	Independent
			Sp1	Human	27	Independent
			HKR1-4	Human	This work	*GLI*-related
C_2H_2-*GLI*	Most fingers	(Y/F)XCX$_3$GCX$_3$(F/Y)X$_5$LX$_2$HX$_{3-4}$H(T/S)GEKP	*GLI*	Human	31	Independent
			MGLI	Mouse	31	*GLI*-related
			GLI2,3	Human	This work	*GLI*-related

Krüppel subgroups on the basis of the features described in Fig. 3 and Table 2. Perhaps reflecting the large size of the zinc finger gene family, characterization of genes from various species has resulted in the isolation of homologs only rarely. Of the genes listed in Table 2, apparent homologs include only (i) *NGFI-A* (rat) and *EGR-1* (mouse) (36, 52) and (ii) *GLI* (human) and *MGLI* (mouse) (31).

There were several distinct differences between the C_2H_2-*GLI* and C_2H_2-*Kr* subclasses. First, the spacing of amino acids between the invariant cysteine and histidine residues for C_2H_2-*GLI* ($CX_3GC\ldots HX_{3-4}H$) differed from C_2H_2-*Kr* ($CX_2C\ldots HXRXH$). Second, exons of the *GLI* subgroup contained only one complete finger, while exons of the Krüppel subgroup contained several fingers. It has previously been noted that most of the nine fingers of *TFIIIA* are contained within individual exons (57). This intron-exon organization of the *TFIIIA* and *GLI* subgroup genes gives support to the hypothesis that finger proteins have evolved by gene duplication of a small subunit encoding one finger of approximately 30 amino acids (37). Another difference was the conservation of amino acid sequence in the finger regions. Although the fingers of *HKR1* to *4* were almost invariant with respect to certain amino acids common to all Krüppel family fingers (Fig. 3B), there were variable amino acids at other positions, so that each of the finger domains within *HKR1* to *4* was unique. This suggests that the DNA-binding specificities or affinities or both would vary considerably among the different proteins encoded by these loci. In contrast, sequences within the finger regions were highly conserved among the *GLI* genes (Fig. 3A). If *GLI* is a sequence-specific DNA-binding transcription factor, as has been demonstrated for a growing list of finger proteins (e.g., *TFIIIA* [15], *SP1* [14], and human glucocorticoid receptor [25]), then the remarkable similarity of finger sequences among *GLI*, *GLI2*, and *GLI3* suggest that each may bind to the same or similar sequences. The study of amino acid variation in fingers binding similar sequences may further the understanding of sequence-specific binding. Notably, sequence variations between *GLI*, *MGLI*, *GLI2*, and *GLI3* are not found at those finger positions proposed to make sequence-specific contacts with DNA (i.e., those amino acids near the conserved leucine; 3).

Consistent with their putative role in nucleic acid binding and transcriptional regulation, various finger proteins have been shown to be involved in important biological processes. Hunchback (55) and snail (5) play key roles in *Drosophila* development, and the retinoic acid receptor may be important in morphogenesis of the chick limb (21, 43, 56). Some members of the *GLI*-Krüppel family have been implicated in the processes of embryonic development and neoplasia (31, 44, 47, 49); the transcription activator *Sp1* acts on a variety of gene promoters in normal tissues (13, 27). Further study of the members of the *GLI*-Krüppel gene family described here may prove important to the understanding of transcriptional mechanisms or differential gene expression or both and may be relevant to both normal and pathological states in humans.

ACKNOWLEDGMENTS

We thank A. Preisinger and J. Desilets for technical assistance, P. White for help in preparing the manuscript, and L. Chan for providing an APO E clone. This work was supported by Public Health Service training grants GM-07184 (to J.M.R.) and CA-09243 (to K.W.K.) from the National Cancer Institute and Public Health Service grants CA-43722 and NS-20023 (to S.H.B.), GM-33903 (to M.L.L.), HD-02080 and HD-17449 (to F.T.K. and M.L.L.), and CA-43460 (to B.V.) from the National Institutes of Health.

LITERATURE CITED

1. **Andrews, P. W., I. Damjanov, D. Simon, G. S. Banting, C. Carlin, N. C. Dracopoli, and J. Fogh.** 1984. Pluripotent embryonal carcinoma clones derived from the human teratocarcinoma cell line tera-2. J. Lab. Invest. 50:147–162.
2. **Berg, J. M.** 1986. Potential metal-binding domains in nucleic acid binding proteins. Science 232:485–487.
3. **Berg, J. M.** 1988. A proposed structure for the zinc binding domains from transcription factor IIIA and related proteins. Proc. Natl. Acad. Sci. USA 85:99–102.
4. **Bigner, S. H., J. Mark, and D. D. Bigner.** 1987. Chromosomal progression of malignant human gliomas from biopsy to establishment as permanent lines in vitro. Cancer Genet. Cytogenet. 24:163–176.
5. **Boulay, J. L., C. Dennefeld, and A. Alberga.** 1987. The *Drosophila* developmental gene *snail* encodes a protein with nucleic acid binding fingers. Nature (London) 330:395–398.
6. **Brown, R. S., C. Sander, and P. Argos.** 1985. The primary

structure of transcription factor TFIIIA has 12 consecutive repeats. FEBS Lett. 186:271–274.
7. Chavrier, P., P. Lemaire, O. Revelant, R. Bravo, and P. Charnay. 1988. Characterization of a mouse multigene family that encodes zinc finger structures. Mol. Cell. Biol. 8:1319–1326.
8. Cheung, P., F. Kao, M. L. Law, C. Jones, T. T. Puck, and L. Chan. 1984. Localization of the structural gene for human apolipoprotein A on the long arm of human chromosome 11. Proc. Natl. Acad. Sci. USA 81:508–511.
9. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162:156–159.
10. Chowdhury, K., U. Deutsch, and P. Gruss. 1987. A multigene family encoding several "finger" structures is present and differentially active in mammalian genomes. Cell 48:771–778.
11. Colberg-Poley, A. M., S. D. Voss, K. Chowdhury, and P. Gruss. 1985. Structural analysis of murine genes containing homeo box sequences and their expression in embryonal carcinoma cells. Nature (London) 314:713–718.
12. Coussens, L., T. L. Yang-Feng, Y.-C. Liao, E. Chen, A. Gray, J. McGrath, P. H. Seeburg, T. A. Libermann, J. Schlessinger, U. Francke, A. Levinson, and A. Ullrich. 1985. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with *neu* oncogene. Science 230:1132–1139.
13. Dynan, W., and R. Tjian. 1985. Control of eukaryotic messenger RNA synthesis by sequence-specific DNA-binding proteins. Nature (London) 316:774–778.
14. Dynan, W. S., and R. Tjian. 1983. The promoter-specific transcription factor Sp1 binds to upstream sequences in the SV40 early promoter. Cell 35:79–87.
15. Engelke, D. R., S.-Y. Ng, B. S. Shastry, and R. G. Roeder. 1980. Specific interaction of a purified transcription factor with an internal control region of 5S RNA genes. Cell 19:717–728.
16. Evans, R. M., and S. M. Hollenberg. 1988. Zinc fingers: gilt by association. Cell 52:1–3.
17. Feinberg, A. P., and B. Vogelstein. 1984. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 137:266–267.
18. Fogh, J. 1978. Cultivation, characterization, and identification of human tumor cells with emphasis on kidney, testis, and bladder tumors. Natl. Cancer Inst. Monogr. 49:5–9.
19. Gehring, W. J. 1985. Homeotic genes, the homeo box, and the genetic control of development. Cold Spring Harbor Symp. Quant. Biol. 50:243–252.
20. Gehring, W. J. 1987. Homeo boxes in the study of development. Science 236:1245–1252.
21. Giguere, V., E. S. Ong, P. Segui, and R. M. Evans. 1987. Identification of a receptor for the morphogen retinoic acid. Nature (London) 330:624–629.
22. Giguere, V., N. Yang, P. Segui, and R. M. Evans. 1988. Identification of a new class of steroid hormone receptors. Nature (London) 331:91–94.
23. Hanas, J. S., D. J. Hazuda, D. F. Bogenhagen, F. Y.-H. Wu, and C.-W. Wu. 1983. *Xenopus* transcription factor A requires zinc for binding to the 5S RNA gene. J. Biol. Chem. 258:14120–14125.
24. Hartshorne, T. A., H. Blumberg, and E. T. Young. 1986. Sequence homology of the yeast regulatory protein ADR1 with Xenopus transcription factor TFIIIA. Nature (London) 320:283–287.
25. Hollenberg, S. M., V. Giguere, P. Segui, and R. M. Evans. 1987. Colocalization of DNA-binding and transcriptional activation functions in the human glucocorticoid receptor. Cell 49:39–46.
25a. Humphrey, P. A., A. J. Wong, B. Vogelstein, H. S. Friedman, M. H. Werner, D. D. Bigner, and S. H. Bigner. 1988. Amplification and expression of the epidermal growth factor receptor gene in human glioma xeno grafts. Cancer Res. 48:2231–2238.
26. Johnson, D. A., J. W. Gautsch, J. R. Sportsman, and J. H. Elder. 1984. Gene analysis techniques. Gene Anal. Tech. 1:308.
27. Kadonaga, J. T., K. R. Carner, F. R. Masiarz, and R. Tijian. 1987. Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. Cell 51:1079–1090.
28. Kao, F., J. A. Hartz, M. L. Law, and J. N. Davidson. 1982. Isolation and chromosomal localization of unique DNA sequences from a human genomic library. Proc. Natl. Acad. Sci. USA 79:865–869.
29. King, C. R., M. H. Kraus, and S. A. Aaronson. 1985. Amplification of a novel v-*erb*B-related gene in a human mammary carcinoma. Science 229:974–976.
30. Kinzler, K. W., S. H. Bigner, D. D. Bigner, J. M. Trent, M. L. Law, S. J. O'Brien, A. J. Wong, and B. Vogelstein. 1987. Identification of an amplified, highly expressed gene in a human glioma. Science 236:70–73.
31. Kinzler, K. W., J. M. Ruppert, S. H. Bigner, and B. Vogelstein. 1988. The Gli gene is a member of the Kruppel family of zinc finger proteins. Nature (London) 332:371–374.
32. Klug, A., and D. Rhodes. 1987. 'Zinc fingers': a novel protein motif for nucleic acid recognition. Trends Biochem. Sci. 12:464–469.
33. Kohl, N. E., N. Kanda, R. R. Schreck, G. Bruns, S. A. Latt, F. Gilbert, and F. W. Alt. 1983. Transposition and amplification of oncogene-related sequences in human neuroblastomas. Cell 35:359–367.
34. Levine, M., G. M. Rubin, and R. Tjian. 1984. Human DNA sequences homologous to a protein coding region conserved between homeotic genes of Drosophila. Cell 38:667–673.
35. McGinnis, W., C. P. Hart, W. J. Gehring, and F. H. Ruddle. 1984. Molecular cloning and chromosome mapping of a mouse DNA sequence homologous to homeotic genes of Drosophila. Cell 38:675–680.
36. Milbrandt, J. 1987. A nerve growth factor-induced gene encodes a possible transcriptional regulatory factor. Science 238:797–799.
37. Miller, J., A. D. McLachlan, and A. Klug. 1985. Repetitive zinc-binding domains in the protein transcription factor IIIA from Xenopus oocytes. EMBO J. 4:1609–1614.
38. Nau, M. M., B. J. Brooks, J. Battey, E. Sausville, A. F. Gazdar, I. R. Kirsch, O.W. McBride, V. Bertness, G. F. Hollis, and J. D. Minna. 1985. L-*myc*, a new *myc*-related gene amplified and expressed in human small cell lung cancer. Nature (London) 318:69–73.
39. O'Brien, S. J., W. G. Nash, J. L. Goodwin, D. R. Lowry, and E. H. Chang. 1983. Dispersion of the *ras* family of transforming genes to four different chromosomes in man. Nature (London) 302:839–842.
40. S. J. O'Brien, T. I. Bonner, M. Cohen, C. O'Connell, and W. G. Nash. 1983. Mapping of an endogenous retroviral sequence to human chromosome 18. Nature (London) 303:74–77.
41. Ollo, R., and T. Maniatis. 1987. *Drosophila* Kruppel gene product produced in a baculovirus expression system is a nuclear phosphoprotein that binds to DNA. Proc. Natl. Acad. Sci. USA 84:5700–5704.
42. Page, D. C., R. Mosher, E. M. Simpson, E. M. C. Fisher, G. Mardon, J. Pollack, B. McGillivray, A. de la Chapelle, and L. G. Brown. 1987. The sex-determining region of the human Y chromosome encodes a finger protein. Cell 51:1091–1104.
43. Petkovich, M., N. J. Brand, A. Krust, and P. Chambon. 1987. A human retinoic acid receptor which belongs to the family of nuclear receptors. Nature (London) 330:444–450.
44. Preiss, A., U. B. Rosenberg, A. Kienlin, E. Seifert, and H. Jackle. 1985. Molecular genetics of *Kruppel*, a gene required for segmentation of the *Drosophila* embryo. Nature (London) 313:27–32.
45. Reed, K. C., and D. A. Mann. 1985. Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res. 13:7207–7221.
46. Rosenberg, U. B., C. Schroder, A. Preiss, A. Kienlin, S. Cote, I. Riede, and H. Jackle. 1986. Structural homology of the product of the Drosophila *Kruppel* gene with Xenopus transcription factor IIIA. Nature (London) 319:336–339.
47. Ruiz i Altaba, A., H. Perry-O'Keefe, and D. A. Melton. 1987. *Xfin*: an embryonic gene encoding a multifingered protein in *Xenopus*. EMBO. J. 6:3065–3070.
48. Russel, M., S. Kidd, and M. R. Kelley. 1986. An improved

filamentous helper phage for generating single-stranded plasmid DNA. Gene 45:333–338.
49. **Schuh, R., W. Aicher, U. Gaul, S. Cote, A. Preiss, D. Maier, E. Seifert, U. Nauber, C. Schroder, R. Kemler, and H. Jackle.** 1986. A conserved family of nuclear proteins containing structural elements of the finger protein encoded by *Kruppel*, a Drosophila segmentation gene. Cell 47:1025–1032.
50. **Schwab, M., K. Alitalo, K. H. Klempnauer, H. E. Varmus, J. M. Bishop, F. Gilbert, G. Brodeur, M. Goldstein, and J. Trent.** 1983. Amplified DNA with limited homology to *myc* cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. Nature (London) 305:245–248.
51. **Shapiro, M. B., and P. Senapathy.** 1987. RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression. Nucleic Acids Res. 15: 7155–7174.
52. **Sukhatme, V. P., X. Cao, L. C. Chang, C.-H. Tsai-Morris, D. Stamenkovich, P. C. P. Ferreira, D. R. Cohen, S. A. Edwards, T. B. Shows, T. Curran, M. M. Le Beau, and E. D. Adamson.** 1988. A zinc finger-encoding gene coregulated with c-fos during growth and differentiation, and after cellular depolarization. Cell 53:37–43.
53. **Tabor, S., and C. C. Richardson.** 1987. DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. Proc. Natl. Acad. Sci. USA 84:4767–4771.
54. **Taparowsky, E., K. Shimizu, M. Goldfarb, and M. Wigler.** 1983. Structure and activation of the human N-*ras* gene. Cell 34:581–586.
55. **Tautz, D., R. Lehmann, H. Schnurch, R. Schuh, E. Seifert, A. Kienlin, K. Jones, and H. Jackle.** 1987. Finger protein of novel structure encoded by *hunchback*, a second member of the gap class of *Drosophila* segmentation genes. Nature (London) 327: 383–389.
56. **Thaller, C., and G. Eichele.** 1987. Identification and spatial distribution of retinoids in the developing chick limb bud. Nature (London) 327:625–628.
57. **Tso, J. Y., D. J. Van Den Berg, and L. J. Korn.** 1986. Structure of the gene for *Xenopus* transcription factor TFIIIA. Nucleic Acids Res. 14:2187–2200.
58. **Vincent, A.** 1986. TFIIIA and homologous genes. The 'finger' proteins. Nucleic Acids Res. 14:4385–4391.
59. **Vincent, A., H. V. Colot, and M. Rosbash.** 1985. Sequence and structure of the *Serendipity* locus of *Drosophila melanogaster*. A densely transcribed region including a blastoderm-specific gene. J. Mol. Biol. 186:149–166.
60. **Vogelstein, B.** 1987. Rapid purification of DNA from agarose gels by centrifugation through a disposable plastic column. Anal. Biochem. 160:115–118.
61. **Vogelstein, B., E. R. Fearon, S. R. Hamilton, A. C. Preisinger, H. F. Willard, A. M. Michelson, A. D. Riggs, and S. H. Orkin.** 1987. Clonal analysis using recombinant DNA probes from the X-chromosome. Cancer Res. 47:4806–4813.
62. **Wingender, E.** 1988. Compilation of transcription regulating proteins. Nucleic Acids Res. 16:1879–1902.
63. **Winter, E., F. Yamamoto, C. Almoguera, and M. Perucho.** 1985. A method to detect and characterize point mutations in transcribed genes: amplification and overexpression of the mutant c-Ki-*ras* allele in human tumor cells. Proc. Natl. Acad. Sci. USA 82:7575–7579.

STIC-ILL

5/10

From: Lacourciere, Karen
Sent: Friday, May 10, 2002 3:43 PM
To: STIC-ILL
Subject: ill order

39578

Please provide the following references for use in examining 09/898,556

Mol. Cell. Biol. (1988), 8(8), 3104-13

Kagaku to Seibutsu (1995), 33(3), 146-8

Fudan Xuebao, Ziran Kexueban (2000), 39(6), 692-694

JOURNAL OF BACTERIOLOGY, (1994 Mar) 176 (5) 1488-99.

JOURNAL OF BACTERIOLOGY, (1996 Jan) 178 (2) 477-83.

GENE, (1998 Nov 5) 222 (1) 61-7.

MOLECULAR AND GENERAL GENETICS, (1999 Mar) 261 (2) 275-80.

ANIMAL GENETICS, (2001 Dec) 32 (6) 375-9.

Thank-you!

MYG 5/13

Karen A. Lacourciere Ph.D.
CM1 11D09 GAU 1635
(703) 308-7523

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

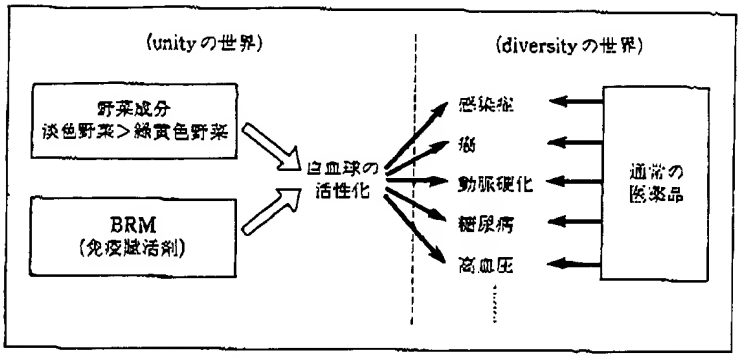

図 1 ■野菜による白血球活性化と疾病改善

ツ，ナス，ダイコンといった淡色野菜が強い TNF 誘導能を示し，その強さは，臨床で用いられている BRM に匹敵するほどである．キュウリ，タマネギといった淡色野菜も，ホウレンソウやニンジンなどの緑黄色野菜と同程度の強さを示す．一方，ピーマンやアオジソといった緑黄色野菜は，白血球活性化能がほとんどない．この白血球活性化の序列は，カロチンやビタミンの序列とはまったく関係ないといえる．

緑黄色野菜が世の中で強調されているが，実は淡色野菜の有効性を指摘する研究も少なくない．たとえば，癌の疫学調査でも，カリフラワー，キャベツ，タマネギ，ナスなどの淡色野菜も有効性が報告されている．それなのになぜ緑黄色野菜だけが良いようになってしまうのだろうか?! 加美山博士らの報告でも，キャベツ，タマネギ，ダイコン，レタスといった淡色野菜は，ニンジンと同程度の変異原性抑制を示す．ちなみに，ピーマン，パセリといった緑黄色野菜は抑制効果を示さなかった．小滝水博士らによるイニシエーション抑制，プロモーション抑制物質の検索でも，ゴボウ，ショウガをはじめとして緑黄色野菜以外の野菜にも，活性物質が広く分布していることが報告されている．このように，緑黄色野菜は必ずしも万能ではないと思われる．体に良いのは，決して緑黄色野菜だけではないはずである．世の中は，イメージとビタミン学に支配され，緑黄色野菜が強調され過ぎていないだろうか？

最近，白血球は感染症や癌だけではなく，種々の成人病と関わり合いをもつことがわかってきた（図 1）．たとえば，動脈硬化に関係する変性脂質やコレステロールは，マクロファージがその代謝に大きな意味をもっている．すなわち，マクロファージを活性化すれば，脂質代謝の調節を通じ病気の改善，予防が期待できる．実際，実験モデルの結果はその考えを支持している[4]．先に，野菜をよく摂取する人は成人病の罹患率が低いと述べた．このことも，おそらく野菜の成分が白血球を適度に活性化し，成人病の予防，改善に関与している局面があると想定される．通常の薬は，個々の病状に焦点をあてた個々の薬である．このような diversity の世界に対し，白血球という unity を中心に据え，病気のコントロールを考えることも大切であろう（図 1）．そして野菜という食べ物は，その unity の世界に通じる大切な道具といえよう．

以上述べてきたように，緑黄色野菜は万能ではなく，淡色野菜も重要であること，食品成分はその直接作用に加え，体の細胞との薬理（食理？）的作用の解明も大切であることをふまえ，白血球という unity を中心に野菜の作用の良さを追求すべきと思われる．

1) 小滝水弘一：ファルマシア，29，604 (1993).
2) M. Yamazaki, H. Ueda, K. Fukuda, *et al.*: *Biosci. Biotech. Biochem.*, 56, 149 (1992).
3) M. Yamazaki & T. Nishimura: *Biosci. Biotech. Biochem.*, 56, 150 (1992).
4) T. Okutomi, T. Nishizawa, H. Inagawa, *et al.*: *Chem. Pharm. Bull.*, 40, 1268 (1992).

（山崎正利，上田浩史，帝京大学薬学部）

酵母のキラートキシン(HM-1)耐性遺伝子とその機能

細胞壁β-1,3-グルカン合成系に関与

真菌（酵母・糸状菌）の細胞壁骨格多糖である β-1,3-グルカンの生合成機構は複雑で，未だ β-1,3-グルカン合成酵素の完全精製はなされておらず，タンパク質化学的・酵素化学的性質の詳細はよく知られていない．現段階では，膜に結合した粗酵素[1]，あるいは膜を可溶化し部分精製した酵素[2]について若干の性質が調べられて

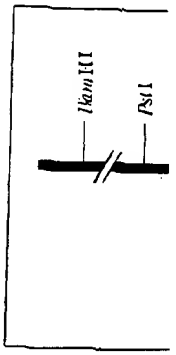

図 1 ■ *Hansenula*
●：*N*-グリコンド型

おり，その結果に
一の酵素タンパク
ク質が関与してい
れば，β-1,3-グル
スに結合する触媒
節サブユニットが
筆者らが *Han*
(HM-1) に注目し
Saccharomyces ce
異的に阻害する点
合成機構を解明す
性質を明らかにす
るが，その酵素タ
以上，β-1,3-グル
その突破口を開く
kii キラートキシン
S. cerevisiae に耐性
1,3-グルカン生合成
換言すると，β-1,3
は β-1,3-グルカン
コードすると仮定し
研究開始にあたり
て多コピーで HM-
離を試みた．キラー
クリーニングは，*S.*
＋トルベクターであ
Sau 3AI で部分消化
を挿入し，ライブラ

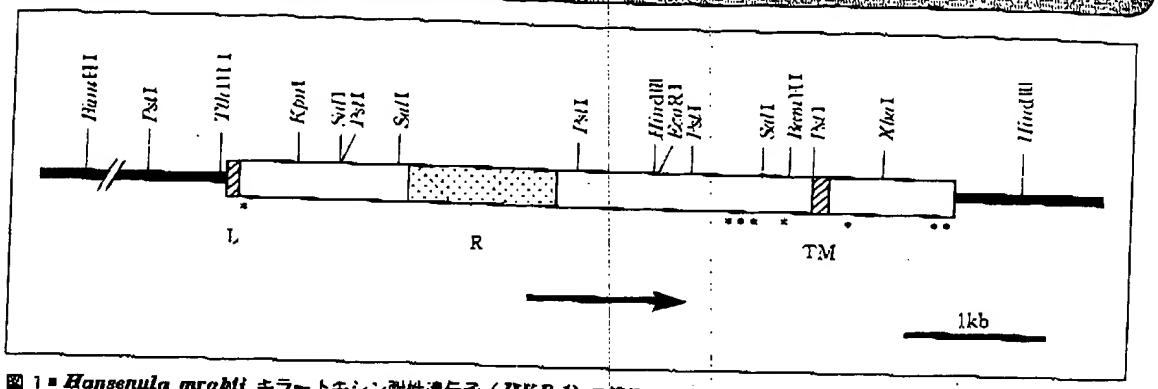

図 1 ■ *Hansenula mrakii* キラートキシン耐性遺伝子（*HKR 1*）の構造
●：*N*-グリコシド型糖鎖結合位置，L：リーダー配列，R：繰り返し配列，TM：膜貫通ドメイン

おり，その結果によれば，β-1, 3-グルカンの生合成は単一の酵素タンパク質による反応ではなく，数種のタンパク質が関与していると予想される．なお，Cabib らによれば，β-1, 3-グルカン合成酵素は，基質 UDP-グルコースに結合する触媒サブユニットと，GTP に結合する調節サブユニットが複合体を形成しているという[5]．

筆者らが *Hansenura mrakii* のキラートキシン（HM-1）に注目したのは，このトキシンの作用が酵母 *Saccharomyces cerevisiae* の β-1, 3-グルカンの合成を特異的に阻害する点にある[4]．酵母の β-1, 3-グルカンの合成機構を解明するためには，前述の酵素タンパク質の性質を明らかにすることから始めるのが常道と考えられるが，その酵素タンパク質の精製がきわめて困難である以上，β-1, 3-グルカン合成に関与する遺伝子から，まずその突破口を開くことを考えた．そこで，まず *H. mrakii* キラートキシン（HM-1）に対して，感受性酵母，*S. cerevisiae* に耐性の形質を付与する遺伝子は，上記 β-1, 3-グルカン生合成系のどこかに機能するタンパク質，換言すると，β-1, 3-グルカン合成酵素そのもの，あるいは β-1, 3-グルカン合成酵素活性を制御する因子などをコードすると仮定した．

研究開始にあたり，*S. cerevisiae* より感受性菌に対して多コピーで HM-1 耐性の表現型を与える遺伝子の単離を試みた．キラートキシン耐性遺伝子（*HKR 1*）のスクリーニングは，*S. cerevisiae* と *Escherichia coli* のシャトルベクターである YEp 213 の *Bam* HI 部位に，*Sau* 3AI で部分消化した *S. cerevisiae* のゲノム DNA を挿入し，ライブラリーとした．これを HM-1 感受性の *S. cerevisiae* A 451 にトランスフェクションして特定の遺伝子の過剰発現を行なった．一方，*S. cerevisiae* A 451 が HM-1 で生育が阻止される濃度（0.7 μg/ml）の寒天培地上で生育できるクローンを選別した．

得られた遺伝子は図 1 に示すように，5,406 塩基対よりなり，1,802 残基のアミノ酸，分子量 189 kDa のタンパク質をコードしている．推定アミノ酸配列から，8 個のアスパラギン結合型糖鎖付加部位が認められた．ハイドロパシープロットの結果によると，N 末端側にシグナルペプチド様の疎水性に富むリーダー配列が存在し，C 末端側には膜貫通領域と思われる非常に疎水性の高い配列がみられる．また，本遺伝子の特徴として中央付近に繰り返し配列があり，さらにカルシウム結合コンセンサス配列として知られている EF ハンドモチーフが認められる．アミノ酸組成の面からも，セリン，スレオニンが全アミノ酸の 36% を占めるかなり特徴的なタンパク質構造をもつものと考えられる．ホモロジー検索の結果，既知のいずれの遺伝子とも異なるものであったが，相同性が最も高い既知遺伝子として *S. cerevisiae* の細胞周期変異（*cdc 24*）のマルチコピーサプレッサー遺伝子（*MSB 2*）が検索され，ヌクレオチドのレベルで約 45%，アミノ酸のレベルで約 33% の相同性が認められた[6]．

さてこの遺伝子（*HKR 1*）はどのような機能をもつタンパク質をコードしているのであろうか．*HKR 1* をガラクトースにより誘導できるプロモーター，GAL 1 あるいは GAL 7 の下流に連結して誘導発現を行なったところ，興味深いことに，ノーザン，ウェスタン両ブロットともに，*HKR 1* 全長を発現させた場合より，C 末端

側（*Hin*d III-*Hin*d III，2.6 kb 断片，図1参照）で強く誘導発現（濃いバンド）がみられた．そこでC末端側フラグメントを過剰発現したときの，*S. cerevisiae* 細胞壁の β-グルカンへの影響を調べると，β-グルカンの含量は顕著に増大し，化学構造的には，β-1,6 結合に対する β-1,3 結合の割合が減少した．β-1,3-グルカン合成酵素活性の変化をみると，*HKR1* の過剰発現により上記酵素活性の低下がみられたことにより，β-1,3-結合の減少という結果が裏づけられた．

HKR1 を *LEU2* 遺伝子で置き換えることによって遺伝子破壊を行ない，胞子の四分子分析で表現型を調べたところ，*HKR1* の破壊は致死となることがわかり，本遺伝子が *S. cerevisiae* の生育に必須であることが証明された[5]．

では，*HKR1* の過剰発現による耐性獲得はどのような機構でなされるのであろうか．感受性酵母 *S. cerevisiae* A 451 に対する HM-1 トキシン作用に及ぼす各種細胞壁類縁多糖の阻害効果を調べると，β-1,3-グルカンが最大の阻害効果を示し，次に β-1,6-グルカンがその約2分の1の効果を示した[6]．この結果は，HM-1 トキシンが酵母細胞壁の β-1,3-グルコシドあるいは β-1,6-グルコシド結合の連鎖部位に親和性があることを示している．

以上の結果を総合すると，次のように考えることができよう．まず耐性遺伝子（*HKR1*）の過剰発現が酵母細胞壁 β-1,3-グルカン合成酵素活性を抑え，その結果 β-1,3-グルカンの構造変化，すなわち β-1,3-グルコシド結合の減少と β-1,6-グルコシド結合の相対的な増加をもたらす．この β-1,3-グルコシド結合の減少は，HM-1 トキシンの結合部位の減少となって現われ，最終的に HM-1 トキシン耐性の表型型を示すことになる．結局，*HKR1* の産物は β-1,3-グルカン合成の制御因子として働いていると考えられる．

1) E. M. Shematak, J. A. Bretz & E. Cabib : *J. Biol. Chem.*, 255, 888 (1980).
2) P. Awald, M. Zugel, C. Monks, D. Frost & C. P. Selitrennikoff : *Exp. Mycol.*, 17, 130 (1993).
3) M. S. Kang & E. Cabib : *Proc. Natl. Acad. Sci. USA*, 83, 5808 (1986).
4) T. Yamamoto, T. Hiratani, H. Hirata, M. Imai & H. Yamaguchi : *FEBS Lett.*, 197, 50 (1986).
5) S. Kasahara, H. Yamada, T. Mio, Y. Shiratori, C. Miyamoto, T. Yabe, T. Nakajima, E. Ichishima & Y. Furuichi : *J. Bacteriol.*, 176, 1488 (1994).
6) S. Kasahara, S. Inoue, T. Mio, T. Yamada, T. Nakajima, E. Ichishima, Y. Furuichi & H. Yamada : *FEBS Lett.*, 348, 27 (1994).

（中島　佑，東北大学農学部）

カラフル・ポテトのアントシアニンに抗酸化作用

未利用遺伝資源の隠された機能を探る

第二次世界大戦後のいわゆる団塊の世代と呼ばれる昭和 20 年代に生を享けた筆者は，現在 40 代半ばを過ぎ，退職後の年金生活を考えて，俸給の何割かを生命保険に掛けている身です．それで俸給日には給与袋を通して世の中の一端を知るようになりました．自動的に共済の積立金，所得税，住民税を天引きされる額が年々大きくなって給与袋が軽くなるにつれて，国民医療費など国・地方財政支出負担の大きさがバカにならないことが感じられます．年齢の2乗か3乗に比例して医者にかかる率が高くなるといわれ，今夏の成人病検査では要精密検査の該当者になり，日常の食事を少しでも考えて健康を維持増進したいと思うのです．

そんな中高年の人口構成比が増えたこともあってでしょうか，農水省の研究プロジェクト「新需要創出」，別名バイオ・ルネッサンス計画の一環として，全国各地域の農畜水産物からこれまで未利用であった有用成分（特に抗酸化性など健康に良い機能性成分）の含量や組成比を育種操作により，増大させたり，改良する研究が平成3年度から進行中です．

ところで，6月下旬から7月中旬に北海道の畑作地帯を訪れた人なら，白（紅丸や農林1号），淡赤紫（男爵薯）や赤紫色（メークイン）の花が一面に咲き乱れるジャガイモ畑の美しさにすぐ気づくのではないでしょうか．このジャガイモの花に魅せられて，18 世紀フランスのルイ 16 世の王妃マリ・アントワネットが髪飾りとして使ったと言われるだけのことはあります．ちなみに現在，

STIC-ILL

GAU 1635
QR1. J8

From: Lacourciere, Karen
Sent: Friday, May 10, 2002 3:43 PM
To: STIC-ILL
Subject: ill order

Please provide the following references for use in examining 09/898,556

Mol. Cell. Biol. (1988), 8(8), 3104-13

Kagaku to Seibutsu (1995), 33(3), 146-8

Fudan Xuebao, Ziran Kexueban (2000), 39(6), 692-694

JOURNAL OF BACTERIOLOGY, (1994 Mar) 176 (5) 1488-99.

JOURNAL OF BACTERIOLOGY, (1996 Jan) 178 (2) 477-83.

GENE, (1998 Nov 5) 222 (1) 61-7.

MOLECULAR AND GENERAL GENETICS, (1999 Mar) 261 (2) 275-80.

ANIMAL GENETICS, (2001 Dec) 32 (6) 375-9.

Thank-you!

Karen A. Lacourciere Ph.D.
CM1 11D09 GAU 1635
(703) 308-7523

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

JOURNAL OF BACTERIOLOGY, Mar. 1994, p. 1488–1499
0021-9193/94/$04.00+0

Vol. 176, No. 5

Cloning of the *Saccharomyces cerevisiae* Gene Whose Overexpression Overcomes the Effects of HM-1 Killer Toxin, Which Inhibits β-Glucan Synthesis

SHIN KASAHARA,[1,2] HISAFUMI YAMADA,[2] TOSHIYUKI MIO,[2] YASUHIKO SHIRATORI,[2] CHIKARA MIYAMOTO,[2] TOMIO YABE,[1] TASUKU NAKAJIMA,[1] EIJI ICHISHIMA,[1] AND YASUHIRO FURUICHI[2]*

Department of Applied Biological Chemistry, Faculty of Agriculture, Tohoku University, Sendai,[1] and Department of Molecular Genetics, Nippon Roche Research Center, Kamakura,[2] Japan

Received 24 September 1993/Accepted 31 December 1993

A gene whose overexpression can endow *Saccharomyces cerevisiae* cells with resistance to HM-1 killer toxin was cloned from an *S. cerevisiae* genomic library. This gene, designated *HKR1* (*Hansenula mrakii* killer toxin-resistant gene *1*), contains a 5.4-kb open reading frame. The predicted amino acid sequence of the protein specified by *HKR1* indicates that the protein consists of 1,802 amino acids and is very rich in serine and threonine, which could serve as O-glycosylation sites. The protein also contains two hydrophobic domains at the N-terminal end and in the C-terminal half, which could function as a signal peptide and transmembrane domain, respectively. Hkr1p is found to contain an EF hand motif of the calcium-binding consensus sequence in the C-terminal cytoplasmic domain. Thus, Hkr1p is expected to be a calcium-binding, glycosylated type I membrane protein. Southern and Northern (RNA) analyses demonstrated that there is a single copy of the *HKR1* gene in the *S. cerevisiae* genome, and the transcriptional level of *HKR1* is extremely low. Gene disruption followed by tetrad analysis showed that *HKR1* is an essential gene. Overexpression of the truncated *HKR1* encoding the C-terminal half of Hkr1p made the cells more resistant to HM-1 killer toxin than the full-length *HKR1* did, demonstrating that the C-terminal half of Hkr1p is essential for overcoming the effect of HM-1 killer toxin. Furthermore, overexpression of *HKR1* increased the β-glucan content in the cell wall without affecting in vitro β-glucan synthase activity, suggesting that *HKR1* regulates β-glucan synthesis in vivo.

The yeast *Hansenula mrakii* secretes a protein with a small molecular mass (10.7 kDa) which kills *Saccharomyces cerevisiae* and other sensitive strains of yeasts. This protein, designated HM-1 killer toxin, consists of 88 amino acids, of which 10 amino acids are cysteine. HM-1 killer toxin is stable in a wide range of pHs (pH 2 to 11) and is heat labile but is sensitive to proteinases and reducing reagents (30). It has also been demonstrated that HM-1 toxin inhibited β-1,3-glucan synthesis in vivo when applied to yeast culture, whereas the synthesis of other cell wall components, such as chitin, mannan, and alkali-soluble glucan, was unaffected (31). In contrast to β-1,3-glucan synthesis, DNA, RNA, protein, and lipid syntheses were apparently not inhibited during the early period of toxin treatment (30). These results implied that HM-1 killer toxin specifically inhibits β-1,3-glucan synthesis and kills the targeted cells by an as yet unknown mechanism. Despite the strong cytocidal effect on *S. cerevisiae* cells which occurs at about 1 μg/ml, HM-1 toxin was weakly inhibitory to β-1,3-glucan synthase activity in the membrane fraction of *S. cerevisiae*; a higher concentration of HM-1 toxin (10 to 100 μg/ml) was needed to inhibit β-1,3-glucan synthase (30).

Concerning β-1,3-glucan synthesis, neither the enzyme(s) nor its gene has been isolated. The enzyme activity of β-1,3-glucan synthesis was detected in crude membrane fractions of *S. cerevisiae* cells and other fungal cells (14, 26), indicating that the enzyme exists in the membrane. It has also been demonstrated that ATP or GTP was required for enzyme activity (20, 27). Since its substrate (UDP-glucose) and activators (GTP and ATP) are available only in the cytoplasm, the active site of the enzyme would be in the cytoplasmic domain, and the nascent glucan may be transported to the cell wall, perhaps through a channel-like enzyme complex. In this context, Kang and Cabib (14) reported that glucan synthesis of *Hansenula anomala* and *Neurospora crassa* consisted of at least two components, a membrane-bound catalytic component and a cytosolic regulatory component, of which the latter could be solubilized by salt and detergent and may represent an affinity for GTP and ATP.

Beside β-1,3-glucan, there is another type of glucan polymer classified as β-1,6-glucan. β-1,6-Glucan accounts for only a small percentage of total β-glucan, while the ratio of β-1,3- and β-1,6-glucan varies among yeast species and under different growth conditions and stages (11). The synthesis of β-1,6-glucan apparently occurs on the long β-1,3-glucan chain via β-1,6-linkage of glucose, which gives rise to a cell wall glucan network (11). Although the mechanism underlying β-1,6-glucan synthesis and its regulation have not been fully understood, Bussey's group has succeeded in isolating various *S. cerevisiae* mutants defective in β-1.6-glucan synthesis. These mutants, designated *kre* mutants, were obtained by screening the *S. cerevisiae* cells that survived in the presence of K1 killer toxin, whose receptor has been identified as cell wall β-1,6-glucan (8, 12). The *kre* mutants which lack the ability to synthesize the normal level of β-1,6-glucan (and therefore acquired a K1 toxin-resistant phenotype) were used for cloning the genes involved in β-1,6-glucan synthesis. Genes whose introduction into *kre* mutants led to the normal level of β-1,6-glucan synthesis and to the loss of resistance to K1 killer toxin were elegantly cloned and characterized (4–7, 12, 19, 22).

In an attempt to clarify the mechanism of action of HM-1

* Corresponding author. Mailing address: Department of Molecular Genetics, Nippon Roche Research Center, 200 Kajiwara, Kamakura, Kanagawa 247, Japan.

and to clone β-1,3-glucan synthesis-associated genes, we have used an expression cloning strategy with HM-1 killer toxin. Here we report the cloning of a gene whose overexpression gave rise to an HM-1 killer toxin-resistant phenotype in *S. cerevisiae*. A possible involvement of this gene product in β-1,3-glucan synthesis is discussed.

MATERIALS AND METHODS

Purification of HM-1 killer toxin. HM-1 killer toxin was purified from the culture medium of *H. mrakii* (IFO0895) by the method of Yamamoto et al. (31) with some modifications. A portion (1/100 volume) of the culture of *H. mrakii* grown overnight was inoculated in several liters of minimal medium containing 0.67% yeast nitrogen base (Difco) and 0.5% glucose. After 30 h, the culture of *H. mrakii* was centrifuged at 4,500 rpm for 10 min with a Kontron A6.9 rotor to remove cells. The supernatant was filtered through a cellulose acetate filter (pore size, 0.45 μm; Corning) to remove cell debris and aggregates. Filtered medium was concentrated by ultrafiltration with Filtron Omega Minisette (nominal molecular mass limit, 3 kDa; Filtron) and then with a YM2 filter (Amicon). HM-1 killer toxin was purified by Sephadex G-50 column chromatography. Several milliliters of the concentrated medium was applied to a Sephadex G-50 column and eluted with 50 mM NaH_2PO_4. Fractions containing HM-1 were combined, and HM-1 was further purified by high-pressure liquid chromatography. The partially purified toxin was loaded on an SP column (SP-2SW; 4.6 mm by 25 cm; Tosoh) and eluted with 25 mM sodium phosphate buffer (pH 5.8) with a linear gradient of 0 to 0.5 M NaCl. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and subsequent staining of the proteins with Coomassie brilliant blue revealed that the purified HM-1 was homogenous, and no visible contamination was detected.

The activity of HM-1 was determined on the basis of the growth inhibition of *S. cerevisiae* cells (strain A451) in synthetic medium containing glucose (SG) (0.17% yeast nitrogen base, 0.5% ammonium sulfate, 2% glucose) supplemented with required amino acids, 20 μg of uracil per ml, and 40 μg of adenine sulfate per ml. Part (1/100 volume) of a culture of *S. cerevisiae* cells grown overnight was inoculated into several milliliters of SG containing various concentrations of HM-1 killer toxin. After inoculation, cell growth was monitored by measuring A_{600} as a function of time.

Construction of a *S. cerevisiae* genomic DNA library. Wild-type *S. cerevisiae* (strain YNN295) genomic DNA purchased from Clontech was partially digested with *Sau*3AI and fractionated on a 0.5% agarose gel. The DNA fragments between 4 and 8 kb long were eluted electrophoretically from the gel (25) and purified with an Elutip-D column (Schleicher & Schuell). The vector used for constructing the library was YEp213, a derivative of YEp13, which carries a 2μm replication origin and *LEU2* gene as a genetic marker (1, 23). *S. cerevisiae* genomic DNA, partially digested with *Sau*3AI and ligated to the *Bam*HI cleavage site of YEp213, and the resulting plasmids were transfected to *Escherichia coli* DH5 competent cells. Plasmid DNA was extracted from the transformed *E. coli* cells through cesium chloride gradient centrifugation as described elsewhere (25).

DNA transfection and screening. *S. cerevisiae* cells (strain A451 *MATα can1 leu2 trp1 ura3 aro7*) were transfected with plasmid DNA by the lithium acetate method (13). For selecting the HM-1-resistant clones, cells were transfected with the genomic DNA library, and were seeded on SG agar plates that lacked leucine but contained 0.7 μg of HM-1 per ml. After incubation at 30°C for 3 to 4 days, plasmid DNA was recovered from the colonies growing on the HM-1-containing plates by lysing the cells with glass beads, extracting with phenol, and precipitating with ethanol (17). The insert DNA was excised by digesting the recovered plasmid DNA with various restriction endonucleases and subcloned in pUC18 and pUC19 vectors. DNA sequencing was performed with Sequenase version 2 kit (USB) and [α-^{35}S]dCTP (NEN).

For obtaining the full-length gene, *S. cerevisiae* genomic DNA was digested with *Bam*HI, and resulting DNA fragments between 5.5 and 8 kb long were purified, ligated at the *Bam*HI cleavage site of the pUC18 vector, transfected into *E. coli* DH5, and screened by colony hybridization with a radiolabelled 1-kb *Hin*dIII-*Bam*HI fragment of the cloned gene. Hybridization and washing of the filters were carried out under stringent conditions (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–1× Denhardt's solution–20 mM sodium phosphate buffer [pH 6.5]–0.1% SDS–50% formamide at 42°C for hybridization; 0.1× SSC–0.1% SDS at 60°C for washing) (25).

Southern and Northern (RNA) blotting. For the preparation of genomic DNA, *S. cerevisiae* cells were treated with Zymolyase 20T and the resulting spheroplasts were lysed by adding SDS to the cell suspension to give a final concentration of 0.1% (17). Cell debris was removed by centrifugation after the addition of potassium acetate. DNA was precipitated with 2-propanol, treated with pancreatic RNase, and digested with endonucleases as indicated. RNA was extracted from growing *S. cerevisiae* cells by lysing the cells with glass beads in the presence of SDS followed by phenol extraction and ethanol precipitation (25). Poly(A)$^+$ RNA was purified from total RNA with Oligotex-$(dT)_{30}$ as described previously (16). Endonuclease-digested DNA and poly(A)$^+$ RNA were fractionated by agarose gel electrophoresis, transferred to nylon membranes, hybridized with radiolabelled probes, and visualized by autoradiography. Radiolabelling of DNA probes was carried out by random-priming methods with [α-^{32}P]dCTP (25). Conditions for hybridization and washing of the filters were identical to those described for colony hybridization.

Overexpression of *HKR1*. In order to overexpress *HKR1*, a 5.9-kb *Tth*111I-*Hin*dIII fragment or a 2.6-kb *Hin*dIII-*Hin*dIII fragment which contains the full-length *HKR1* gene and part of the gene encoding the C-terminal part of Hkr1p, respectively, were ligated at the *Bgl*II cleavage site of pMT34-317, a derivative of pMT34 (28). These plasmids were designated pMT-*HKR* and pMT-*HKR*^{tr} (tr for truncated), respectively. In these plasmids, *HKR1* gene transcription was under control of the *GAL7* promoter. After transformation of *S. cerevisiae* A451 cells with these plasmid DNAs, uracil prototrophs were collected, analyzed, and used for the experiments. Induction of *HKR1* gene expression was carried out as described previously (28). Part (1/100 volume) of a culture of the cells grown overnight in glucose-containing medium was inoculated into synthetic medium containing 2% galactose, and the cells were further incubated at 30°C.

Disruption of the *HKR1* gene. The strains carrying null mutations of *HKR1* were generated by single-step gene disruption (24). A plasmid required for homologous recombination was constructed by replacing the *Kpn*I-*Xba*I region of *HKR1* with the *S. cerevisiae LEU2* gene. The chimeric *HKR1-LEU2* gene was then excised from the plasmid vector and used to transform a diploid *S. cerevisiae* strain (RAY3A-D a/α *ura3/ura3 leu2/leu2 his3/his3 trp1/trp1*). To confirm that the disrupted copy of *HKR1* had been integrated at the expected chromosomal locus in the diploid strain, genomic DNA was isolated from several leucine prototrophs and then digested

with *Eco*RV and *Hin*dIII f llowed by Southern blot analysis with a 0.9-kb *Xba*I-*Hin*dIII fragment of *HKR1* as a probe, which would give rise to a 5.7-kb normal *HKR1* allele and a 3.2-kb *HKR1-LEU2* chimeric allele.

Preparation of cell wall glucan. Cell wall polysaccharides were fractionated from *S. cerevisiae* A451 cells transformed with pMT34-317, pMT-*HKR*^{tr}, or pMT-*HKR* by the methods of Peat et al. (21) and Manners et al. (18) with some modifications. Lyophilized *S. cerevisiae* cells grown to late logarithmic phase in synthetic medium containing galactose and lacking uracil were autoclaved for 90 min at 120°C. The insoluble residues were collected by centrifugation and extracted four times with 1.0 N NaOH containing 0.5% $NaBH_4$ for 24 h at 30°C with gentle shaking. After centrifugation, the supernatant fractions containing alkali-soluble glucans were neutralized with acetic acid, dialyzed against H_2O, and lyophilized. The precipitates were also neutralized and extracted five times with 0.5 M acetic acid at 90°C for 90 min. The acid-insoluble glucans were centrifuged, dialyzed against H_2O, and lyophilized. The carbohydrate content of each fraction was estimated by the phenol-sulfuric acid method by using glucose as a standard (10).

RESULTS

Cloning of a gene with multicopy suppression activity of HM-1 killer toxin. In an attempt to clone *S. cerevisiae* genes involved in β-1,3-glucan synthesis, we employed an expression cloning approach using HM-1 killer toxin, which has been implicated as an inhibitor of β-1,3-glucan synthesis. Overproduction of the protein which might bind to toxin was anticipated to protect the cells from the lethal effect of HM-1 killer toxin. To this end, the minimal concentration of HM-1 required for its lethal effects on *S. cerevisiae* A451 was first examined. After serial dilutions of purified HM-1 toxin and inclusion in the *S. cerevisiae* proliferation assay, the threshold of the lethal concentration was estimated to be 0.5 μg/ml in agar plates. To ensure the growth arrest of the untransfected cells, a slightly higher concentration (0.7 μg/ml) of HM-1 was used for the screening. A genomic DNA library was constructed with a YEp213 vector which had a 2μm replication origin. The library contained partially *Sau*3AI-digested genomic DNA that was between 4 and 8 kb long. *S. cerevisiae* A451 cells were transformed with this library, and the transformants were then seeded on the leucine-depleted SG agar plates which contained the lethal dose (0.7 μg/ml) of the purified HM-1 killer toxin. Of 10^5 transformants, 10 colonies appeared after 4 days of incubation at 30°C. Cells from these colonies were grown in SG lacking leucine and were subjected to a secondary screening. Of 10 clones, 6 showed healthy growth, even in the presence of 0.7 μg of HM-1 killer toxin per ml. Plasmid DNA was extracted from cells derived from the six clones, and a restriction enzyme map of the insert DNA was determined. The size (7.3 kb) and restriction map of the insert DNA was found to be identical among the six clones, indicating that all of the HM-1-resistant clones contained the same genomic DNA fragment. Since the introduction of vector plasmid (YEp213) alone did not alter the sensitivity of the cells to the toxin, it was evident that this DNA fragment was essential for resistance to HM-1 killer toxin (Fig. 1A). Next, we have analyzed the essential region of the insert DNA fragment for the HM-1-resistant phenotype. Transformation of *S. cerevisiae* cells with DNA fragments which had been digested with various end nucleases revealed that the 2.6-kb *Hin*dIII-*Hin*dIII regi n located near the 5′ end f the 7.3-kb DNA fragment was sufficient t make the cells resistant to 0.7 μg of

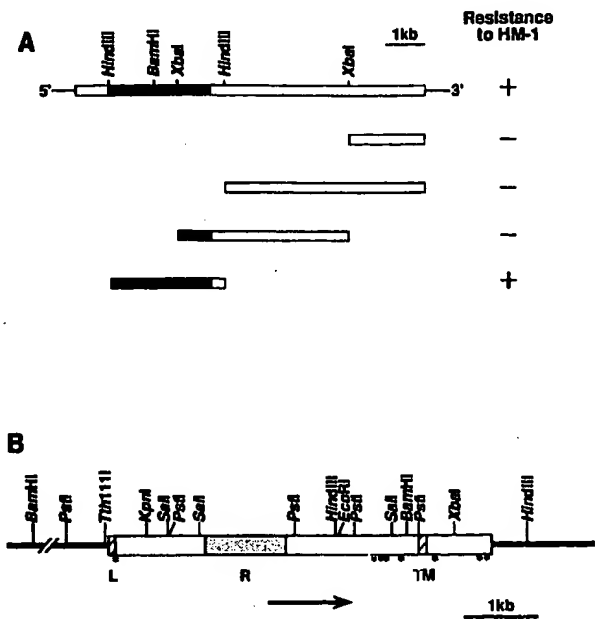

FIG. 1. Restriction map of *HKR1* and the essential region for resistance to HM-1. (A) Plasmid DNA was isolated from the HM-1-resistant cells, and the insert DNA was digested with various endonucleases. The resulting DNA fragments were subcloned into the YEp213 vector, transfected to A451 cells, and tested for the ability to overcome growth inhibition by 0.7 μg of HM-1 per ml. The solid box indicates an open reading frame found in this DNA fragment. The open box represents the coding region of *HKR1*. (B) Predicted structure of Hkr1p. R, repetitive sequence; L, possible leader sequence; TM, transmembrane domain. Asterisks indicate potential N glycosylation sites.

HM-1 per ml (Fig. 1A). A more detailed study indicated that the A451 cells harboring the 2.6-kb *Hin*dIII-*Hin*dIII fragment were resistant up to 10 μg of HM-1 killer toxin per ml (data not shown).

The sequencing of this 2.6-kb *Hin*dIII-*Hin*dIII fragment demonstrated that there was an open reading frame which was capable of coding for a protein with an approximate molecular mass of 73 kDa. We designated this gene *HKR1* (*Hansenula* killer toxin-resistant gene *1*). Despite its ability to rescue *S. cerevisiae* cells from 10 μg of HM-1 killer toxin per ml, the 2.6-kb *Hin*dIII-*Hin*dIII fragment seemed to be part of the gene, because the open reading frame could still continue to the 5′ end of the insert DNA. Furthermore, there was no typical promoter sequence, such as a TATA box in the 5′ sequence upstream from the first methionine codon. These observations strongly suggested that the 2.6-kb *Hin*dIII-*Hin*dIII fragment was part of the *HKR1* gene. To address this possibility, we carried out Northern blotting to determine the length of the endogenous mRNA for this gene. Cells transformed with YEp213 carrying the 2.6-kb *Hin*dIII-*Hin*dIII fragment of *HKR1* expressed mRNA of the expected size at a high level (Fig. 2, lane 1), whereas mRNA of the same size was not detected in the cells transformed with the vector alone (Fig. 2). Instead, a larger (about 6-kb-long) mRNA was detected in the cells transformed with *HKR1* and with the vector alone. The level of expression of endogenous *HKR1* was very low; the 6-kb transcript was visible only when m re than 10 μg of poly(A)$^+$ RNA was subjected to N rthern blotting

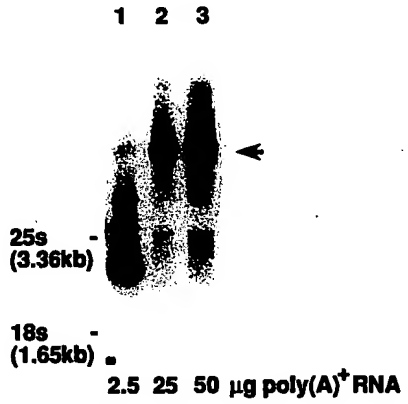

FIG. 2. Northern blot analysis of *HKR1* mRNA. The indicated amount of poly(A)$^+$ RNA was isolated from cells carrying the 2.6-kb *Hin*dIII-*Hin*dIII fragment of *HKR1* in YEp213 (lane 1) or YEp213 alone (lanes 2 and 3), fractionated on an agarose gel, transferred to a nylon membrane, hybridized with ^{32}P-labelled probe, and visualized by autoradiography. The positions for 25S and 18S rRNA are indicated. The arrow indicates the position of the endogenous *HKR1* mRNA.

(Fig. 2, lane 2 and 3). From these results, we concluded that the 2.6-kb *Hin*dIII-*Hin*dIII fragment was part of the *HKR1* gene and that the full gene might be about 6 kb long. Transcription of the 2.6-kb *Hin*dIII-*Hin*dIII fragment of *HKR1* might have started from promoter-like sequences in the YEp213 vector.

Southern blot analysis with genomic DNA revealed that there was a single copy of the *HKR1* gene in the genome and that there was a *Bam*HI site about 6.5 kb 5′ upstream from the *Bam*HI site located near the 5′ end of the cloned DNA fragment (Fig. 3). In order to clone the missing 5′ part of *HKR1*, *S. cerevisiae* genomic DNA was digested with *Bam*HI, and the resulting DNA fragments between 5.5 to 8 kb long

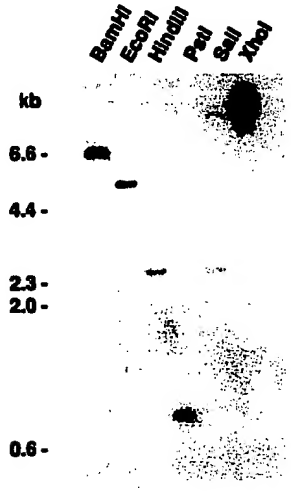

FIG. 3. Genomic Southern blot analysis of *HKR1*. Genomic DNA (5 μg) was digested with *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, *Sal*I, and *Xho*I, fractionated on an agarose gel, transferred to a nylon membrane, hybridized with ^{32}P-labelled probe, and visualized by autoradiography. The positions of size markers are indicated.

were purified from the gel, subcloned into pUC19 vector, and screened with radiolabelled *HKR1* probe. Of 10^4 clones, we obtained two clones which strongly hybridized to the probe. Restriction maps of these two clones were identical. Sequencing of the full-length *HKR1* gene demonstrated that there was a 5.4-kb open reading frame which could encode a protein with an approximate molecular mass of 189 kDa (1,802 amino acids) (Fig. 1B and 4).

The predicted amino acid sequence of the protein specified by *HKR1* (Hkr1p) showed two hydrophobic domains at the N terminus (21 amino acids) and in the C-terminal half (26 amino acids) (Fig. 1B, 4, and 5). According to Von Heijne's criteria (29), it was predicted that the N-terminal 21 amino acids could serve as a signal peptide. The hydrophobic domain in the C-terminal half could be a membrane-spanning domain, since this domain can form seven turns of α-helical structure. Another noteworthy feature of Hkr1p was that this protein was very rich in serine and threonine. Over 30% of the total amino acids were serine and threonine, which might serve as acceptors for O-linked glycosylation. In addition to the possible O-linked glycosylation, there were eight potential N-linked glycosylation sites (Fig. 1B). Repetitive sequences were found in the middle of the protein; there were 12 repeats of the 28 amino acids (Ser-Ala-Pro-Val-Ala-Val-Ser-Ser-Thr-Tyr-Thr-Ser-Ser-Pro-Ser-Ala-Pro-Ala-Ala-Ile-Ser-Ser-Thr-Tyr-Thr-Ser-Ser-Pro) (Fig. 4). Although there has been no gene or protein reported so far whose sequence was identical to that of *HKR1* or Hkr1p, *HKR1* had a significant sequence similarity to *MSB2*, a multicopy suppressor gene of *S. cerevisiae cdc24* (2, 3). Hkr1p shared 33% sequence identity and 45% sequence similarity with Msb2p throughout the proteins. Structural resemblance between Hkr1p and Msb2p was also observed; Msb2p, which consists of 1,306 amino acids, is very rich in serine and threonine and has a signal sequence at the N terminus, a hydrophobic transmembrane domain in the C-terminal half, and finally repetitive sequences in the middle of the protein (3).

Interestingly, Hkr1p had a sequence, Asp-Val-Asp-Glu-Asn-Gly-Asp-Ile-Arg-Leu-Tyr-Asp, starting at position 1645. This sequence strongly correlated with the EF hand motif of the calcium-binding site: Asp (or Asn)-Val-Asp (or Asn)-Glu-Asn (or Asp or Ser)-Gly-Asp-Ile (or Val)-Arg-Leu-Tyr-Asp (or Glu), in which the 1st Asp (or Asn), 3rd Asp (or Asn), 5th Asn (or Asp or Ser), 8th Ile (or Val), and 12th Asp (or Glu) are conserved (Fig. 6). This EF hand motif was originally identified in carp parvalbumin as the calcium-binding site (15) and has been found in several other calcium-binding proteins in a wide variety of organisms, including *S. cerevisiae* and higher eukaryotes.

Part of *HKR1* which could encode only a portion of the C-terminal half of Hkr1p is sufficient to overcome the effect of HM-1. As mentioned above, overexpression of the 2.6-kb *Hin*dIII-*Hin*dIII fragment of *HKR1* which could encode part of the C-terminal half of Hkr1p was sufficient to evade the cytocidal effect of HM-1 (at least up to 10 μg/ml). We also overexpressed the full-length *HKR1* by using the *GAL7* promoter, because the entire *HKR1* gene could not be subcloned in YEp213 for unknown reasons. We succeeded in subcloning the full-length *HKR1* gene in pMT34-317, a vector carrying the *GAL7* promoter and the *URA3* gene as a selectable marker. Either the entire sequence or the 2.6-kb *Hin*dIII-*Hin*dIII fragment of *HKR1* was inserted just downstream of the *GAL7* promoter (designated pMT-*HKR* and pMT-*HKR*^{tr}, respectively). The resulting plasmids were transfected into *S. cerevisiae* cells. Expression of *HKR1* was induced by culturing the Ura3$^+$ cells in the medium containing galactose instead of glucose. As

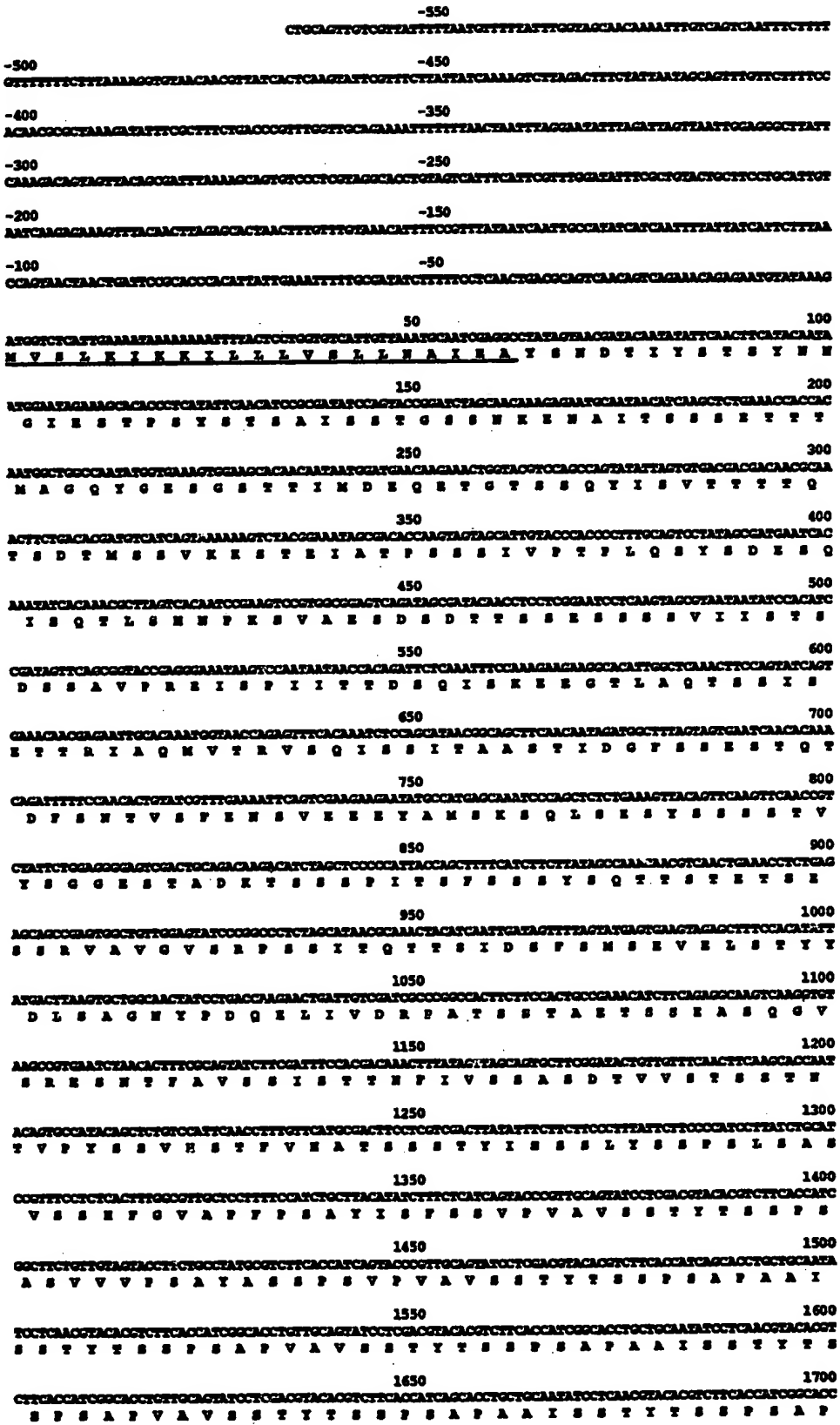

FIG. 4. Nucleotide sequence of *HKR1* and predicted amino acid sequence of Hkr1p. Possible leader sequence and transmembrane domain are indicated by underline and shaded box, respectively.

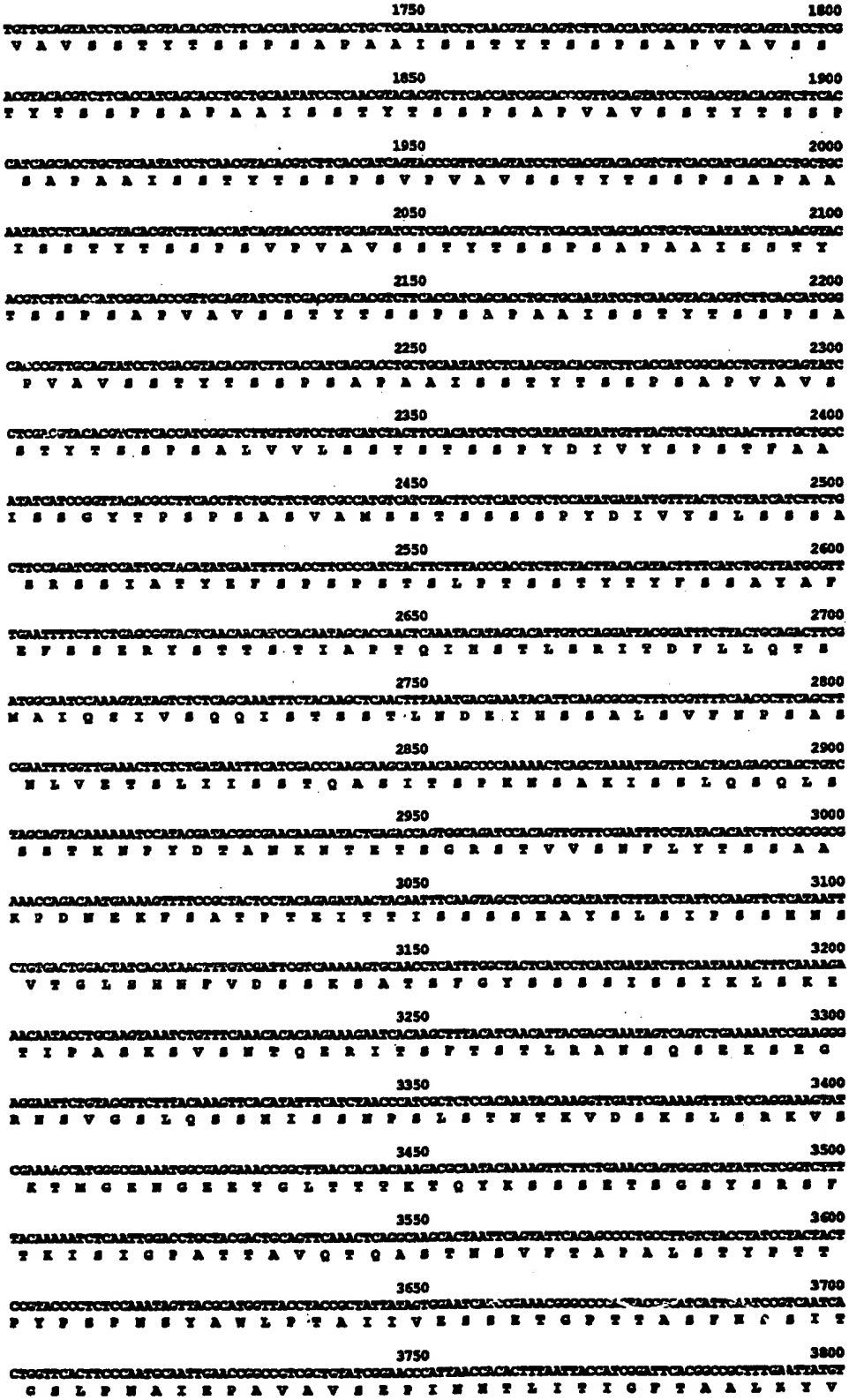

FIG. 4—*Continued.*

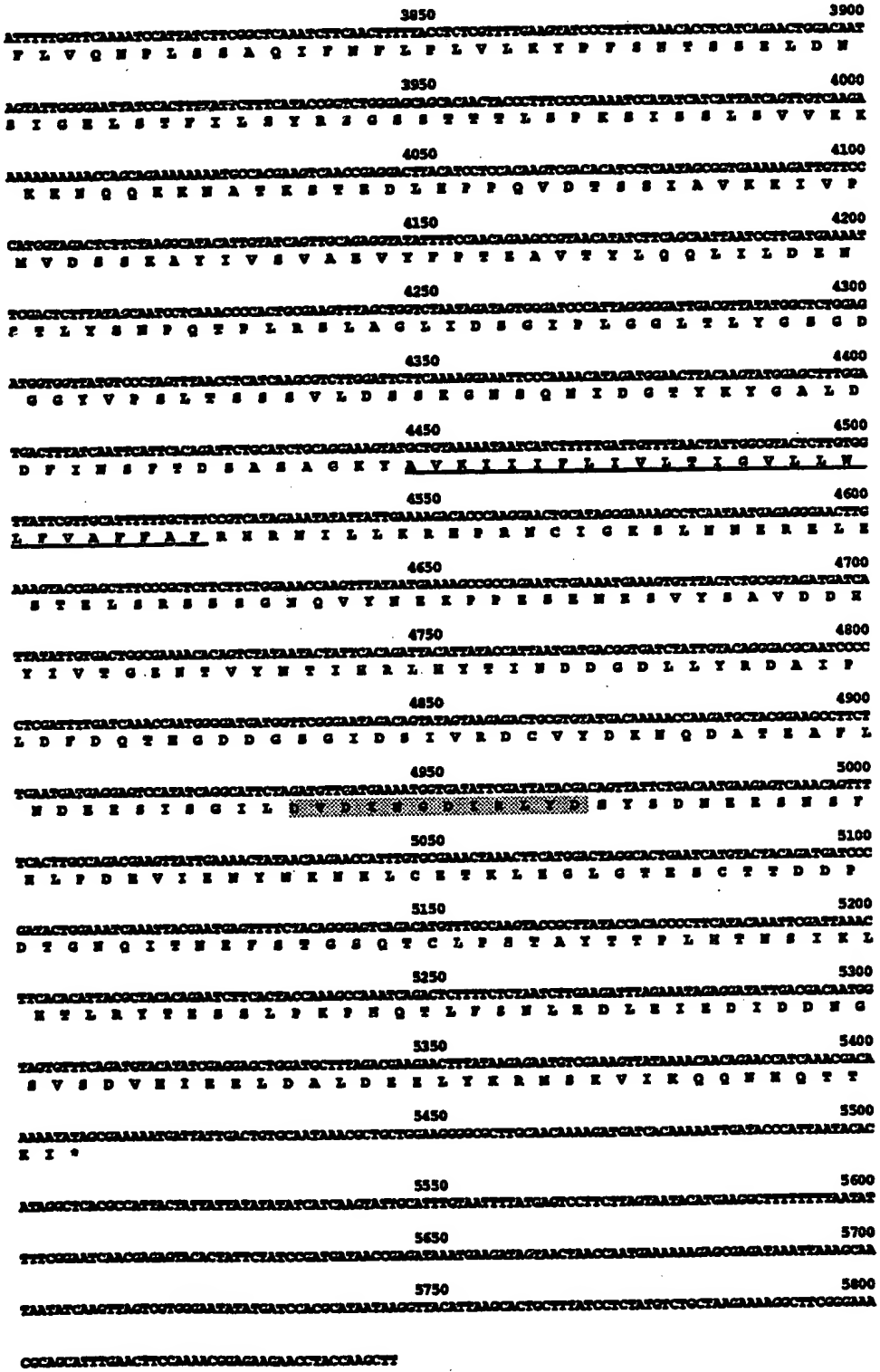

FIG. 4—*Continued.*

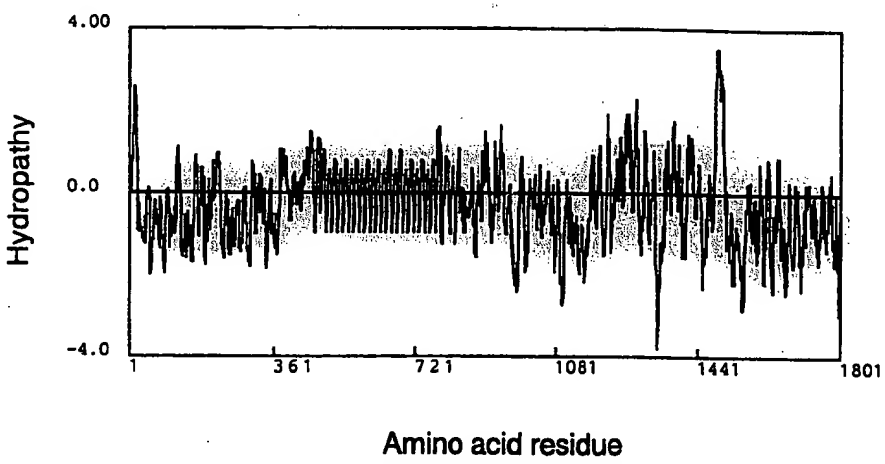

FIG. 5. Hydrophobic profile of Hkr1p. Hydropathy of Hkr1p was calculated by using Kyte and Doolittle parameters (16a) from the predicted amino acid sequence of the protein.

Consensus (EF-hand)		**DXDXNGXIXXXD** **N N D V E** **S**
Hkr1p	1645	**DVDENGDIRLYD**
Troponin C [human]	146	**DKNNDGRIDYDE**
Parvalbumin [human]	56	**DKDKSGFIEEDE**
Calmodulin [*P. hybrida*(plant)]	98	**DKDQNGYISAAD**
Calmodulin [*S. cerevisiae*]	99	**DKNGDGLISAAE**
Cdc31p [*S. cerevisiae*]	147	**DLDGDGEINENE**
H^+ pump [*S. cerevisiae*]	65	**DSDNDGPVAAGE**
Glucanase [*C. thermocellum*(bacterium)]	714	**DVDGNGRINSTD**

FIG. 6. Consensus sequence of the calcium-binding site (EF hand motif) in Hkr1p. The EF hand motif in several calcium-binding proteins and Hkr1p is shown. The amino acids indicated in boldface type are conserved, and X can be any amino acid. The number indicates the amino acid position of the first aspartic acid in this motif. *P. hybrida*, *Petunia hybrida*; *C. thermocellum*, *Clostridium thermocellum*.

shown in Fig. 7, culturing the cells in the galactose-containing medium resulted in more than 50-fold increases in the mRNA levels of both full-length and truncated *HKR1* compared with those of the cells cultured in glucose-containing medium. Then, the effect of HM-1 toxin on the *HKR1*-overexpressing cells was examined by monitoring the growth of these cells in the presence of HM-1. In galactose-containing medium, the cells harboring pMT-*HKR*^{tr} grew normally in the presence of 4 μg of HM-1 per ml (Fig. 8A). Overexpression of the full-length *HKR1* in galactose-containing medium delayed the onset of growth but resulted in normal growth of the cells in the presence of 2 μg of toxin per ml. Full-length *HKR1*, however, did not make the cells resistant to higher concentrations of the toxin; they grew poorly in the presence of 4 μg of HM-1 per ml

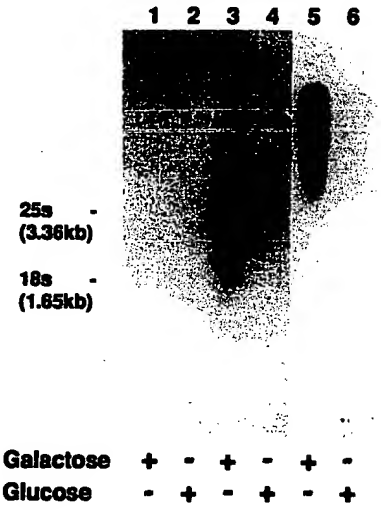

FIG. 7. Induction of *HKR1* gene expression by the *GAL7* promoter. Samples (2 μg) of poly(A)$^+$ RNA from cells carrying pMT34-317 (lanes 1 and 2), pMT-*HKR*^{tr} (lanes 3 and 4), or pMT-*HKR* (lane 5 and 6) which were cultured in galactose- or glucose-containing medium were fractionated on an agarose gel, transferred to a nylon membrane, hybridized with ^{32}P-labelled probe, and visualized by autoradiography. The positions of 25S and 18S rRNA are indicated.

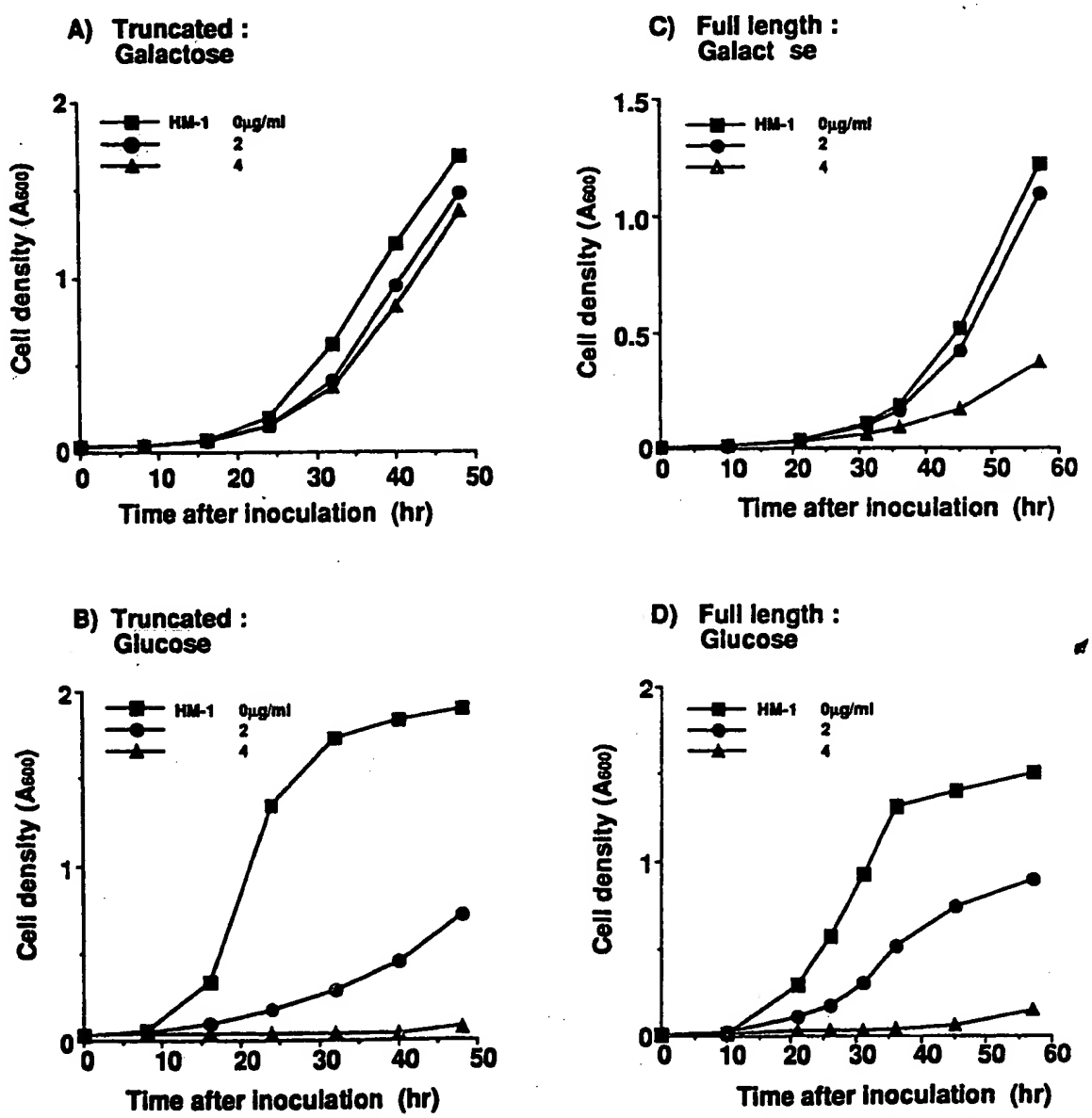

FIG. 8. Effect of *HKR1* overexpression on the growth of cells in the presence of HM-1. Cells transformed with pMT-*HKR*^{tr} (truncated) or pMT-*HKR* (full length) were cultured in glucose- or galactose-containing medium in the presence of the indicated amount of HM-1. Growth of the cells was measured by monitoring A_{600} at various times after inoculation.

(Fig. 8C). Transfection of the vector (pMT34-317) alone did not lead to the cell growth in the presence of HM-1 toxin at all; 0.7 μg of HM-1 per ml was enough to kill the vector-transfected cells in both glucose- and galactose-containing medium (data not shown). When cells were cultured in glucose-containing medium, only low levels of *HKR1* expression were detected, and under this condition, 2 μg of HM-1 per ml almost completely inhibited the growth of cells harboring pMT-*HKR* and pMT-*HKR*^{tr} (Fig. 8B and D). From these results, we concluded that *HKR1* causes the multicopy suppression of HM-1 killer toxin action in *S. cerevisiae* cells and that the C-terminal half f Hkr1p is essential for overcoming the killing effect of HM-1.

***HKR1* is an essential gene.** As described above, the *S. cerevisiae* genome contains a single copy of the *HKR1* gene (Fig. 3). This fact enabled us to examine whether *HKR1* is an essential gene. To address this question, we constructed a plasmid in which most of the coding region of *HKR1* (region between *Kpn*I and *Xba*I sites) was replaced with the *LEU2* gene (Fig. 9A). *S. cerevisiae* cells were transformed with this plasmid, and the *HKR1* disruptants were identified among Leu2$^+$ transf rmants by S uthern blot hybridization with *HKR1* as a probe. Three independent disruptant clones were allowed to sporulate, and the viability of each spore was examined following tetrad dissecti n. We analyzed 20 asci in each three disruptant clones by tetrad dissecti n and found

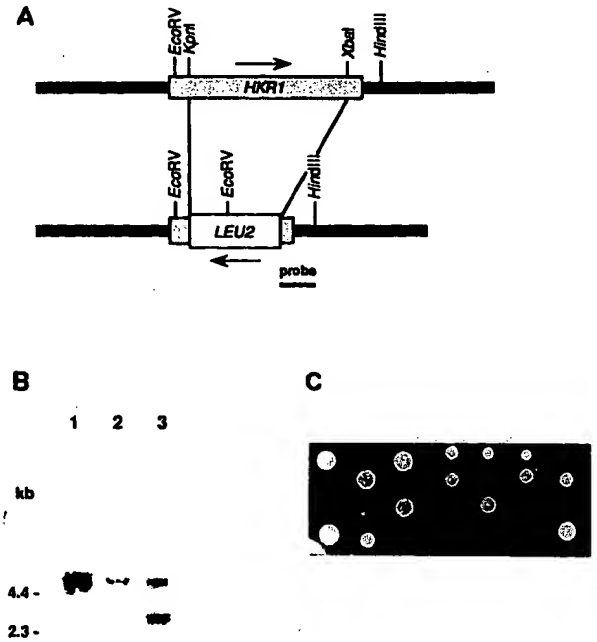

FIG. 9. Disruption of *HKR1* by homologous recombination and subsequent tetrad dissection. (A) The plasmid required for homologous recombination was constructed by replacing the *Kpn*I-*Xba*I region of *HKR1* with a *LEU2* cassette which was inserted in the direction opposite that of *HKR1*. Diploid RAY3A-D cells were transformed with the resulting *HKR1-LEU2* chimeric DNA, and several leucine prototrophs were allowed to sporulate. The probe used for the Southern blotting is indicated. (B) Southern blot analysis of DNA from parental RAY3A-D (lane 1), LEU2$^+$ transformants (lane 3), and haploid cells originated from a viable spore after tetrad dissection (lane 2). The positions of the size markers are indicated. The slower-migrating band corresponds to the normal *HKR1* allele, and the faster-migrating one is attributed to the disrupted allele. (C) Tetrad analysis of *HKR1* disruptants. RAY3A-D clones which were confirmed to contain the disrupted allele of *HKR1* were subjected to tetrad analysis. In most of the cases, only two of four spores were viable.

that only two of four spores were shown to be viable, even after 1 week of cultivation. Furthermore, cells that originated from viable spores were confirmed to contain only an intact allele of *HKR1* (Fig. 9B and C). All these results demonstrated that *HKR1* is an essential gene for the growth of *S. cerevisiae* cells.

Overexpression of *HKR1* increases the cell wall β-glucan content. Since HM-1 killer toxin specifically interferes with β-1,3-glucan synthesis of *S. cerevisiae* (30), it would be of interest to ask whether overexpression of *HKR1* affects β-1,3-glucan synthase activity. This was tested first by β-1,3-glucan synthase assay in vitro (9, 26) with membrane fractions prepared from the cells transformed with pMT-*HKR* or pMT-*HKR*^{tr}. Induction of *HKR1* expression by culturing the cells in the galactose-containing medium did not influence the in vitro β-1,3-glucan synthase activity (data not shown). Next, we examined whether *HKR1* is involved in β-glucan synthesis in

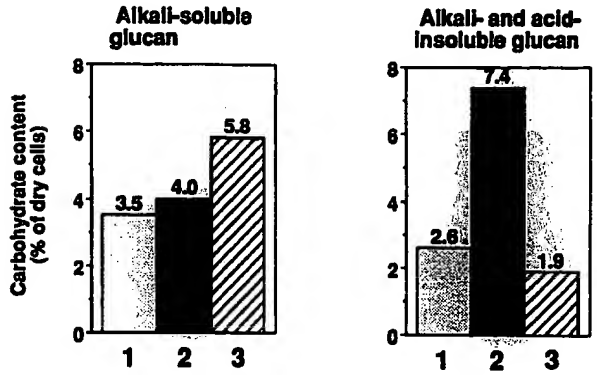

FIG. 10. Changes in β-glucan content by overexpression of *HKR1*. β-Glucan was fractionated from the cells transformed with pMT34-317 (bar 1), pMT-*HKR*^{tr} (bar 2), and pMT-*HKR* (bar 3), and carbohydorate contents in the alkali-soluble glucan fraction and in the alkali- and acid-insoluble glucan fractions were determined by the phenol-sulfuric acid method.

vivo. For this purpose, the β-glucan content of the cell wall was determined in cells overexpressing either the truncated or full-length *HKR1* gene. Comparison of the carbohydorate contents of *HKR1*-overexpressing cells and vector-transfected cells revealed that the overexpression of full-length *HKR1* resulted in about 50% increase in the alkali-soluble β-glucan content and that the alkali- and acid-insoluble β-glucan content was increased by about 2.5-fold by the overexpressed truncated *HKR1* (Fig. 10). Furthermore, most of the carbohydorates both in the alkali-soluble and in the alkali- and acid-insoluble glucan fractions were identified as β-1,3-glucan (not shown). These results demonstrated that the overexpression of either truncated or full-length *HKR1* increased the level of the cell wall β-1,3-glucan and suggest that *HKR1* is involved in β-1,3-glucan biosynthesis.

DISCUSSION

We have isolated a *S. cerevisiae* gene whose overexpression made *S. cerevisiae* cells resistant to HM-1 killer toxin. This gene, designated *HKR1*, encodes a high-molecular-weight protein (the calculated molecular mass is 189 kDa) that has the profile of a type I membrane protein. As mentioned in Results, *HKR1* shares a significant sequence similarity to *MSB2*, a multicopy suppressor of *cdc24* (3). Furthermore, both Hkr1p and Msb2p can code for high-molecular-weight proteins which are rich in serine and threonine and are structurally related; they have a signal sequence at the N terminus, possess a transmembrane domain in the C-terminal half, and contain amino acid repeats in the middle of the proteins (3). These results prompted us to examine whether *HKR1* could substitute for *MSB2* and rescue a *cdc24* mutant at the nonpermissive temperature. To address this question, we carried out a preliminary experiment in which pMT-*HKR* and pMT-*HKR*^{tr} were introduced into *S. cerevisiae* Y147 (*cdc24*) cells (2). *HKR1* mRNA was increased more than 50-fold in cells cultured in galactose-containing medium compared with in the cells cultured in glucose-containing medium, as shown in Fig. 7. This increased level of *HKR1* expression, however, did not support the growth of Y147 cells at 37°C, suggesting that *HKR1* cannot substitute for *MSB2*. *MSB2* has been reported to be a nonessential gene (3), while *HKR1* was shown to be essential for the

viability of *S. cerevisiae* cells. Taking these results together, we concluded that *HKR1* and *MSB2* are functionally distinct.

We found that overexpression of truncated *HKR1*, which could encode the C-terminal part of Hkr1p, was sufficient and much more efficient than the full-length gene in conferring resistance to HM-1 killer toxin. This difference may be caused mainly by the difference in the levels of expressed protein. Indeed, Western blotting (immunoblotting) with a specific antibody raised against the C-terminal portion of Hkr1p showed that the level of truncated Hkr1p was much higher than that of the full-length Hkr1p (data not shown).

It is not clear at present how the full-length or truncated Hkr1p could protect the cells from HM-1 toxin, and further experiments should be necessary to understand the molecular mechanism of HM-1 killer toxin action. Surprisingly, most, if not all, of the protein expressed from the truncated form of *HKR1* (2.6-kb *Hin*dIII-*Hin*dIII region) was found in the membrane fraction (data not shown). One possible interpretation is that Hkr1p contains a HM-1 toxin binding site in the C-terminal half of the protein and that overexpressed Hkr1p neutralizes the HM-1 killer toxin by binding to it.

On the other hand, Hkr1p contains an EF hand motif of the calcium-binding consensus sequence in the C-terminal cytoplasmic domain. The existence of a calcium-binding site in Hkr1p suggests that the changes in the intracellular calcium concentration may be associated with the cytocidal effect by HM-1 toxin. This finding also enables us to speculate that the overexpression of the full-length or truncated form of Hkr1p impairs the function of the endogenous Hkr1p at certain steps, e.g., binding to calcium ion and interaction with substrate.

As for the physiological function of Hkr1p, we demonstrated here that overexpression of *HKR1* increased the β-glucan content and that *HKR1* is involved in β-glucan biosynthesis. β-Glucan synthase activity in the cells, however, was unaffected by the overexpression of either truncated or full-length *HKR1*, suggesting that Hkr1p may be a regulatory factor of β-glucan synthesis. Interestingly, truncated and full-length *HKR1* affected the β-glucan content in different fractions. Since the level of truncated Hkr1p is much higher than that of full-length Hkr1p, the increase in the β-glucan content in the different fractions by the two forms of Hkr1p may also be the consequence of the difference in the protein levels.

ACKNOWLEDGMENTS

We thank J. R. Pringle (University of North Carolina) and Y. Matsui (University of Tokyo) for *S. cerevisiae* Y147 (*cdc24*), F. Hishinuma (Mistubishi Kasei Institute of Life Sciences) for YEp213 and *S. cerevisiae* A451, and K. Mizumoto and Y. Shibagaki (Kitasato University) for pMT34-317, *S. cerevisiae* RAY3A-D, and advice on tetrad analysis. We especially thank T. Yamada (Yokohama City University) for technical assistance and repeated discussions.

This work was supported in part by a grant in aid (Glycotechnology Program) from the Ministry of Agriculture, Forestry and Fisheries of Japan to T.N.

REFERENCES

1. **Andreadis, A., Y.-P. Hsu, G. B. Kohlhaw, and P. Schimmel.** 1982. Nucleotide sequence of yeast *LEU2* shows 5′ noncoding region has sequences cognate to leucine. Cell **31**:319–325.
2. **Bender, A., and J. R. Pringle.** 1989. Multicopy suppression of the *cdc24* budding defect in yeast by *CDC42* and three newly identified genes including the *ras*-related gene *RSR1*. Proc. Natl. Acad. Sci. USA **86**:9976–9980.
3. **Bender, A., and J. R. Pringle.** 1992. A Ser/Thr rich multicopy suppressor of a *cdc24* bud emergence defect. Yeast **8**:315–323.
4. **Boone, C., M. Goebl, R. Puccia, A.-M. Sdicu, and H. Bussey.** 1993. Yeast *KRE2* defines a new gene family encoding probable secretory proteins, and is required for the correct N-glycosylation of proteins. Genetics **130**:273–283.
5. **Boone, C., S. S. Sommer, A. Hensel, and H. Bussey.** 1990. Yeast *KRE* genes provide evidence for a pathway of cell wall β-glucan assembly. J. Cell Biol. **110**:1833–1843.
6. **Brown, J. L., and H. Bussey.** 1993. The yeast *KRE9* encodes an O glycoprotein involved in cell surface β-glucan assembly. Mol. Cell. Biol. **13**:6346–6356.
7. **Brown, J. L., Z. Kossaczka, B. Jiang, and H. Bussey.** 1993. A mutational analysis of killer toxin resistance in *Saccharomyces cerevisiae* identifies new genes in cell wall (1-6)-β-glucan synthesis. Genetics **133**:837–849.
8. **Bussey, H., D. Saville, K. Hutchins, and R. G. E. Palfree.** 1979. Binding of yeast killer toxin to a cell wall receptor on sensitive *Saccharomyces cerevisiae*. J. Bacteriol. **140**:888–892.
9. **Cabib, E., and M. S. Kang.** 1987. Fungal 1,3-β-glucan synthase. Methods Enzymol. **138**:637–642.
10. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350–356.
11. **Fleet, G. H.** 1991. Cell walls, p. 199–277. *In* A. H. Rose and J. S. Harrinson (ed.), The yeasts, vol. 4. Academic Press, New York.
12. **Hutchens, K., and H. Bussey.** 1983. Cell wall receptor for yeast killer toxin: involvement of (1-6)-β-D-glucan. J. Bacteriol. **154**:161–169.
13. **Ito, H., Y. Fukuda, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153**:163–168.
14. **Kang, M. S., and E. Cabib.** 1986. Regulation of fungal cell wall growth: a guanine nucleotide-binding proteinaceous component required for activity of (1,3)-β-D-glucan synthase. Proc. Natl. Acad. Sci. USA **83**:5808–5812.
15. **Kretsinger, R. H., and C. E. Nockolds.** 1973. Carp muscle calcium-binding protein. J. Biol. Chem. **248**:3313–3326.
16. **Kuribayashi-Ohta, K., S. Tamatsukuri, M. Hikata, C. Miyamoto, and Y. Furuichi.** 1993. Application of oligo(dT)$_{30}$-latex for rapid purification of poly(A)$^+$ mRNA and for hybrid subtraction with the in situ reverse transcribed cDNA. Biochim. Biophys. Acta **1156**:204–212.

16a. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157**:105–132.

17. **Lundblad, V.** 1989. *Saccharomyces cerevisiae*, p. 13.11.1–13.11.5. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. Wiley Interscience, New York.
18. **Manners, D. J., A. J. Masson, and J. C. Patterson.** 1973. The structure of a β-(1,3)-D-glucan from yeast cell walls. Biochem. J. **135**:19–30.
19. **Meaden, P., K. Hill, J. Wagner, D. Slipetz, S. S. Somer, and H. Bussey.** 1990. The yeast *KRE5* gene encodes a probable endoplasmic reticulum protein required for (1-6)-β-D-glucan synthesis and normal cell growth. Mol. Cell. Biol. **10**:3013–3019.
20. **Notario, V., H. Kawal, and E. Cabib.** 1982. Interaction between yeast β-(1,3)-glucan synthetase and activating phosphorylated compounds. J. Biol. Chem. **257**:1902–1905.
21. **Peat, S., W. J. Whelan, and T. E. Edwards.** 1958. Polysaccharides of baker's yeast. Part II. Yeast glucan. J. Chem. Soc. **1958**:3862–3868.
22. **Roemer, T., and H. Bussey.** 1991. Yeast β-glucan synthesis: *KRE6* encodes a predicted type II membrane protein required for glucan synthesis *in vivo* and for glucan synthase activity *in vitro*. Proc. Natl. Acad. Sci. USA **88**:11295–11299.
23. **Rose, M. D., F. Winston, and P. Hieter.** 1990. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Rothstein, R.** 1983. One-step gene disruption in yeast. Methods Enzymol. **101**:202–209.
25. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

26. **Shematek, E. M., J. A. Braatz, and E. Cabib.** 1980. Biosynthesis of the yeast cell wall. I. Preparation and property of β-(1,3)-glucan synthetase. J. Biol. Chem. **255**:888–894.
27. **Shematek, E. M., and E. Cabib.** 1980. Biosynthesis of the yeast cell wall. II. Regulation of β-(1,3)-glucan synthetase by ATP and GTP. J. Biol. Chem. **255**:895–902.
28. **Tajima, M., Y. Nogi, and T. Fukasawa.** 1986. Duplicate upstream activating sequences in the promoter region of the *Saccharomyces cerevisiae GAL7* gene. Mol. Cell. Biol. **6**:246–256.
29. **Von Heijne, G.** 1986. A new method for predicting signal sequence cleavage site. Nucleic Acids Res. **14**:4683–4690.
30. **Yamamoto, T., T. Hiratani, H. Hirata, M. Imai, and H. Yamaguchi.** 1986. Killer toxin from *Hansenula mrakii* selectively inhibits cell wall synthesis in a sensitive yeast. FEBS Lett. **197**:50–54.
31. **Yamamoto, T., M. Imai, K. Tachibana, and M. Mayumi.** 1986. Application of monoclonal antibodies to the isolation and characterization of a killer toxin secreted by *Hansenula mrakii*. FEBS Lett. **195**:253–257.

STIC-ILL

NPL

From: Lacourciere, Karen
Sent: Friday, May 10, 2002 3:43 PM
To: STIC-ILL
Subject: ill order

Please provide the following references for use in examining 09/898,556

Mol. Cell. Biol. (1988), 8(8), 3104-13

Kagaku to Seibutsu (1995), 33(3), 146-8

Fudan Xuebao, Ziran Kexueban (2000), 39(6), 692-694

JOURNAL OF BACTERIOLOGY, (1994 Mar) 176 (5) 1488-99.

JOURNAL OF BACTERIOLOGY, (1996 Jan) 178 (2) 477-83.

GENE, (1998 Nov 5) 222 (1) 61-7.

MOLECULAR AND GENERAL GENETICS, (1999 Mar) 261 (2) 275-80.

ANIMAL GENETICS, (2001 Dec) 32 (6) 375-9.

Thank-you!

Karen A. Lacourciere Ph.D.
CM1 11D09 GAU 1635
(703) 308-7523

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

JOURNAL OF BACTERIOLOGY, Jan. 1996, p. 477–483
0021-9193/96/$04.00+0

Vol. 178, No. 2

HKR1 Encodes a Cell Surface Protein That Regulates Both Cell Wall β-Glucan Synthesis and Budding Pattern in the Yeast *Saccharomyces cerevisiae*

TOMIO YABE,[1,2] TOSHIKO YAMADA-OKABE,[3] SHIN KASAHARA,[2]† YASUHIRO FURUICHI,[4] TASUKU NAKAJIMA,[2] EIJI ICHISHIMA,[2] MIKIO ARISAWA,[1] AND HISAFUMI YAMADA-OKABE[1]*

Department of Mycology, Nippon Roche Research Center,[1] and Agene Institute,[4] Kamakura, Department of Applied Biological Chemistry, Faculty of Agriculture, Tohoku University, Sendai,[2] and Department of Hygiene, Yokohama City University School of Medicine, Yokohama,[3] Japan

Received 8 August 1995/Accepted 9 November 1995

We previously isolated the *Saccharomyces cerevisiae HKR1* gene that confers on *S. cerevisiae* cells resistance to HM-1 killer toxin secreted by *Hansenula mrakii* (S. Kasahara, H. Yamada, T. Mio, Y. Shiratori, C. Miyamoto, T. Yabe, T. Nakajima, E. Ichishima, and Y. Furuichi, J. Bacteriol. 176:1488–1499, 1994). *HKR1* encodes a type 1 membrane protein that contains a calcium-binding consensus sequence (EF hand motif) in the cytoplasmic domain. Although the null mutation of *HKR1* is lethal, disruption of the 3′ part of the coding region, which would result in deletion of the cytoplasmic domain of Hkr1p, did not affect the viability of yeast cells. This partial disruption of *HKR1* significantly reduced β-1,3-glucan synthase activity and the amount of β-1,3-glucan in the cell wall and altered the axial budding pattern of haploid cells. Neither chitin synthase activity nor chitin content was significantly affected in the cells harboring the partially disrupted *HKR1* allele. Immunofluorescence microscopy with an antibody raised against Hkr1p expressed in *Escherichia coli* revealed that Hkr1p was predominantly localized on the cell surface. The cell surface localization of Hkr1p required the N-terminal signal sequence because the C-terminal half of Hkr1p was detected uniformly in the cells. These results demonstrate that *HKR1* encodes a cell surface protein that regulates both cell wall β-glucan synthesis and budding pattern and suggest that bud site assembly is somehow related to β-glucan synthesis in *S. cerevisiae*.

β-Glucan is one of the major components of the yeast cell wall and is divided into two categories, β-1,3-linked glucan and β-1,6-linked glucan. Both types of β-glucan are synthesized from UDP-glucose by the enzymes called β-glucan synthases, and they are often linked to each other (for a review, see reference 18).

A putative β-1,3-glucan synthase gene was isolated from a *Saccharomyces cerevisiae* genomic DNA library by complementing the hypersensitive phenotype to immunosuppressants FK506 and cyclosporin A and it was designated *FKS1* (15). *FKS1* is identical to *ETG1*, which complements echinocandin-resistant mutation of *S. cerevisiae* cells (15, 16). More recently, Inoue et al. (21) partially purified the 200-kDa protein that was associated with β-1,3-glucan synthase activity from whole-cell membranes of *S. cerevisiae* and cloned the gene for this protein. This gene, referred to as *GSC1*, is also nearly identical to *FKS1* (also called *ETG1*), and disruption of either *FKS1* (*ETG1*) or *GSC1* drastically decreases β-1,3-glucan synthase activity and confers resistance to echinocandins (15, 21). All these facts strongly support the idea that *FKS1* (also called *ETG1*) or *GSC1* encodes a subunit of β-1,3-glucan synthase.

On the other hand, genes involved in β-1,6-glucan synthesis are isolated by the functional complementation of *kre* mutants that lack β-1,6-glucan and are thereby resistant to K1 killer toxin (4–8, 26, 30, 31). Of several *KRE* genes, *KRE5*, *KRE6*, and *SKN1* are believed to play important roles in the early steps of β-1,6-glucan synthesis because the Kre5p sequence is similar to the UDP-glucose:glycoprotein glucosyltransferase sequence (28), and a putative UDP-glucose binding motif was found in Kre6p and Skn1p (30, 31). Further, the recent report demonstrating that Bgl2p, which was originally identified as an endoglucanase, can link β-1,3-glucan chains with β-1,6 linkage at the higher concentrations of the substrate (19) suggests that Bgl2p is one of the proteins that assemble β-1,3- and β-1,6-glucan.

Previously, we isolated *HKR1*, which rendered *S. cerevisiae* cells less susceptible to HM-1 killer toxin produced by *Hansenula mrakii* (22). *HKR1* is an essential gene and encodes a serine- and threonine-rich type 1 membrane protein with a Ca^{2+}-binding consensus sequence (EF hand motif) in the cytoplasmic domain. Although the physiological role of *HKR1* remains to be established, the facts that the null mutation of *HKR1* is lethal and that overexpression of *HKR1* increases the amount of β-1,3-glucan in the cell wall suggest that *HKR1* is involved in both cell growth and β-1,3-glucan synthesis.

In an attempt to gain further insights into the function of *HKR1*, we disrupted this gene at various sites and found that the partial disruption of *HKR1* that would eliminate the cytoplasmic domain of Hkr1p was not lethal. However, the cells harboring the partially disrupted *HKR1* allele exhibited decreased β-1,3-glucan synthase activity and cell wall β-1,3-glucan level. Furthermore, this partial disruption of *HKR1* altered the axial budding pattern of haploid yeast cells. On the basis of these findings, the mechanism of regulation of β-glucan synthesis and bud site selection by *HKR1* are discussed.

* Corresponding author. Mailing address: Department of Mycology, Nippon Roche Research Center, 200 Kajiwara, Kamakura, Kanagawa 247, Japan. Phone: 81-467-47-2213. Fax: 81-467-46-5320. Electronic mail address: hisafumi.okabe@roche.com.

† Present address: Center for Agricultural Biotechnology, University of Maryland Biotechnology Institute, Baltimore, MD.

MATERIALS AND METHODS

Partial disruption and overexpression of *HKR1*. For the partial disruption of *HKR1*, a 2.6-kb *Hin*dIII-*Hin*dIII fragment of *HKR1* was cloned in pUC18 in which the *Bam*HI site in the polycloning site had been already destroyed to generate pUCHKR1H. Then, the *S. cerevisiae LEU2* gene was ligated at the *Bam*HI cleavage site of pUCHKR1H in the direction opposite that of *HKR1* transcription, and the resulting *HKR1-LEU2* chimeric gene was excised from the vector and used to transform a diploid yeast strain, RAY3A-D (a/α *ura3/ura3 leu2/leu2 his3/his3 trp1/trp1*). After selection on a leucine-depleted plate, several Leu^+ transformants were allowed to sporulate and were dissected for tetrads. Integration of the disrupted copy of *HKR1* at the expected locus was confirmed by Southern blot analysis as described below. The haploid RAY3A-D strains harboring the intact *HKR1* gene and the partially disrupted *HKR1* allele were designated HKR1 and hkr1ΔC, respectively, and they were cultured in YPD medium for further study.

In order to overexpress *HKR1*, haploid A451 (α *can1 leu2 trp1 ura3 aro7*) cells were transformed with pMTHKR1 or $pMTHKR1^{tr}$ in which the entire *HKR1* gene or the 2.6-kb *Hin*dIII-*Hin*dIII fragment of *HKR1* was inserted at the *Bgl*II cleavage site (just downstream of the *GAL7* promoter) of pMT34-317 (22, 37), and *HKR1* expression was induced by culturing the cells in galactose-containing medium as described in a previous study (22).

Southern blotting. Genomic DNA was extracted from *S. cerevisiae* cells by lysing the cells with glass beads followed by phenol extraction and ethanol precipitation (24). Genomic DNA (25 μg) was digested with *Eco*RI, separated on agarose gels, transferred to nylon membranes, hybridized with the radiolabelled probe, and visualized by autoradiography. The probe used for Southern blotting was the 1.0-kb *Eco*RI-*Bam*HI fragment of *HKR1*, which would give rise to a 5.5-kb normal *HKR1* allele and a 1.8-kb *HKR1-LEU2* chimeric allele. Radiolabelling of probes was carried out by the random priming method with [α-^{32}P]dCTP (32). Conditions for hybridization and washing the filters have already been described (22).

Determination of cell wall glucan content. Cell wall polysaccharides were fractionated from HKR1 or hkr1ΔC cells that were generated from RAY3A-D by the methods of Manners et al. (25) with some modifications. Lyophilized cells grown to mid-logarithmic phase in YPD medium were autoclaved for 90 min. The insoluble residues were collected by centrifugation and extracted four times with 1.0 N NaOH containing 0.5% $NaBH_4$ at 30°C for 24 h with gentle shaking. After centrifugation, the supernatant fractions containing alkali-soluble glucans were neutralized with acetic acid, dialyzed against H_2O, and lyophilized. The precipitates were also neutralized and extracted five times with 0.5 M acetic acid at 90°C for 90 min. The acid-insoluble glucan was centrifuged, suspended in H_2O, and lyophilized. The carbohydrate content of each fraction was estimated by the phenol-sulfuric acid method by using glucose as a standard (17).

Determination of cell wall chitin content. Cell wall chitin content was estimated by determining the amount of *N*-acetyl-D-glucosamine. The freeze-dried cells of HKR1 and hkr1ΔC that were harvested in mid-logarithmic phase were suspended in 4 M HCl and boiled for 4 h. The amount of amino sugars in the resulting hydrolysate was determined by the Elson-Morgan method (3), using *N*-acetyl-D-glucosamine (hydrolyzed under the same conditions described above) as a standard. After the resulting hydrolysates were diluted with H_2O to obtain a final HCl concentration of 0.2 M, 1 ml of acetylacetone reagent containing 2% (vol/vol) acetylacetone and 1.25 M Na_2CO_3 was added to the 0.5-ml sample of the diluted hydrolysates, and the resulting solution was incubated at 90°C for 60 min. After the solution was cooled to room temperature, 10 ml of ethanol and 1 ml of Ehrlich's reagent containing 2.67% (wt/vol) *p*-dimethylaminobenzaldehyde, 50% (vol/vol) ethanol, and 6 N HCl were added to the solution, which was then incubated at room temperature for 60 min. Hexosamine content was determined by measuring optical density at 530 nm with a spectrophotometer.

Morphological study. The budding pattern was determined by staining bud scars with Calcofluor White (Sigma) as described previously (29). Results obtained with Calcofluor White were consistently observed in at least four independent experiments.

For electron microscopy, cells in mid-logarithmic phase were fixed in 0.1 M phosphate buffer (pH 7.2) containing 2.5% glutaraldehyde at 4°C overnight, dehydrated through a series of acetone solutions, dried in a critical point drying apparatus (Hitachi HCP-1), coated with platinum in an Eiko IB-3 coating unit, and then examined with a scanning electron microscope (Hitachi S-700).

Assays of chitin and β-glucan synthases. The activities of chitin synthase and β-1,3-glucan synthase were determined by the methods of Cabib et al. (10) and Cabib and Kang (9), respectively. The chitin synthase assay was carried out in a standard 50-μl reaction mixture containing 30 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$, 32 mM GlcNAc, 0.1 mM [^{3}H]UDP-GlcNAc (specific activity, 95,880 dpm/nmol), and 20 μg of proteins from total membranes at 30°C. In some cases, enzymes were treated with trypsin (final concentration, 1 mg/ml [wt/vol]) at 30°C for 15 min and then phenylmethanesulfonyl fluoride (final concentration, 2 mg/ml) was added to terminate the trypsin digestion. The β-glucan synthase assay was performed in a standard 50-μl reaction mixture containing 50 mM Tris-HCl (pH 7.5), 10 mM potassium fluoride, 1 mM EDTA, 0.2 mM [^{14}C]UDP-glucose (specific activity, 705 dpm/pmol), and 20 μg of proteins from total membranes at 30°C. In some experiments, 0.5 mM GTP was added to stimulate the reaction. Both chitin synthase and β-glucan synthase reactions were terminated by adding 10% trichloroacetic acid, and radioactivity incorporated into acid-insoluble chitin or β-glucan fractions was determined with a toluene-based liquid scintillator.

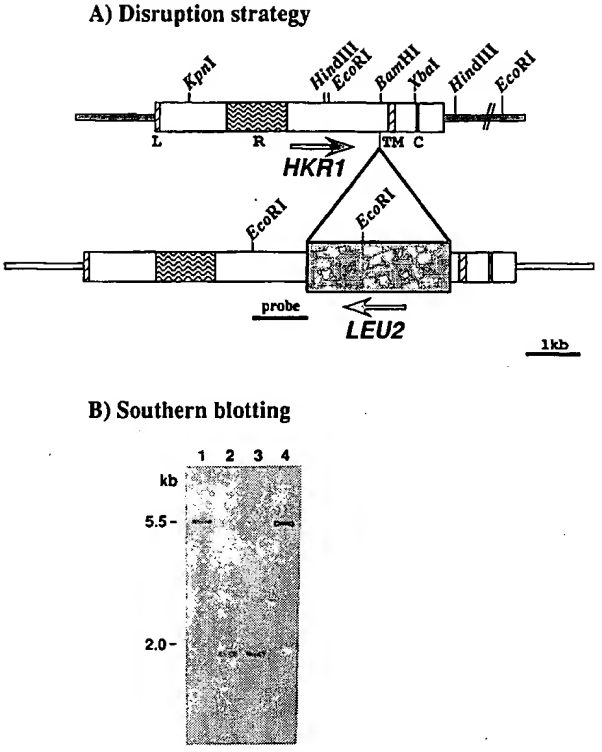

FIG. 1. Partial disruption of *HKR1*. (A) Restriction map of *HKR1* and *HKR1-LEU2* chimeric gene used for homologous recombination. The direction of transcription of *HKR1* and *LEU2* is indicated by the arrows. L, leader sequence; R, repetitive sequence; TM, transmembrane domain; C, calcium-binding EF hand motif. (B) Southern blotting of genomic DNA from haploid HKR1 cells (lane 1), haploid hkr1ΔC cells (lane 2), diploid RAY3A-D cells in which one of the *HKR1* alleles was replaced with the *HKR1-LEU2* chimeric gene (lane 3), and diploid RAY3A-D cells with the intact gene (lane 4). The positions of size markers corresponding to 5.5 and 2.0 kb are indicated to the left of the gel. For more details, see Materials and Methods.

Generation of antibody and Western blotting (immunoblotting). A polyclonal antibody was raised against the C-terminal part of Hkr1p (from amino acid positions 1084 to 1803), which was expressed in *Escherichia coli* cells as a fusion protein with glutathione *S*-transferase (GST). The 0.9-kb *Xba*I-*Hin*dIII fragment of *HKR1* was ligated at the *Eco*RI cleavage site of pGEX2T (Pharmacia), and the resulting plasmid, pGEXHKR1, was introduced into *E. coli* JM109 cells. Expression of GST-Hkr1p fusion protein was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the fusion protein was purified by glutathione Sepharose-4B column chromatography (36). Then Hkr1p was excised by digesting the fusion protein with thrombin and separated from GST by repeating glutathione Sepharose-4B column chromatography. Rabbits were subsequently immunized with the purified Hkr1p, and immunoglobulin G fractions containing anti-Hkr1p antibody were obtained from the crude sera by ammonium sulfate precipitation followed by protein A Sepharose column chromatography (14).

For the Western blotting, the indicated amounts of total membranes or partially purified Hkr1p that was expressed in *E. coli* were fractionated on sodium dodecyl sulfate (SDS)-polyacrylamide gels, transferred to polyvinylidene difluoride membranes electrophoretically (32), incubated with anti-Hkr1p polyclonal antibody and horseradish peroxidase that was conjugated with anti-rabbit immunoglobulin G, and visualized with cyclic diacrylhydrazides (enhanced chemiluminescence detection kit; Amersham).

Immunofluorescence microscopy. Localization of Hkr1p was determined by indirect immunofluorescence microscopy with an anti-Hkr1p polyclonal antibody. A451 cells, which were transformed with pMTHKR1 or $pMTHKR1^{tr}$ and were grown to mid-logarithmic phase, were fixed with 2% formaldehyde solution at room temperature for 60 min and then made permeable by suspension in phosphate-buffered saline (PBS) containing 0.1% (vol/vol) Triton X-100. After the

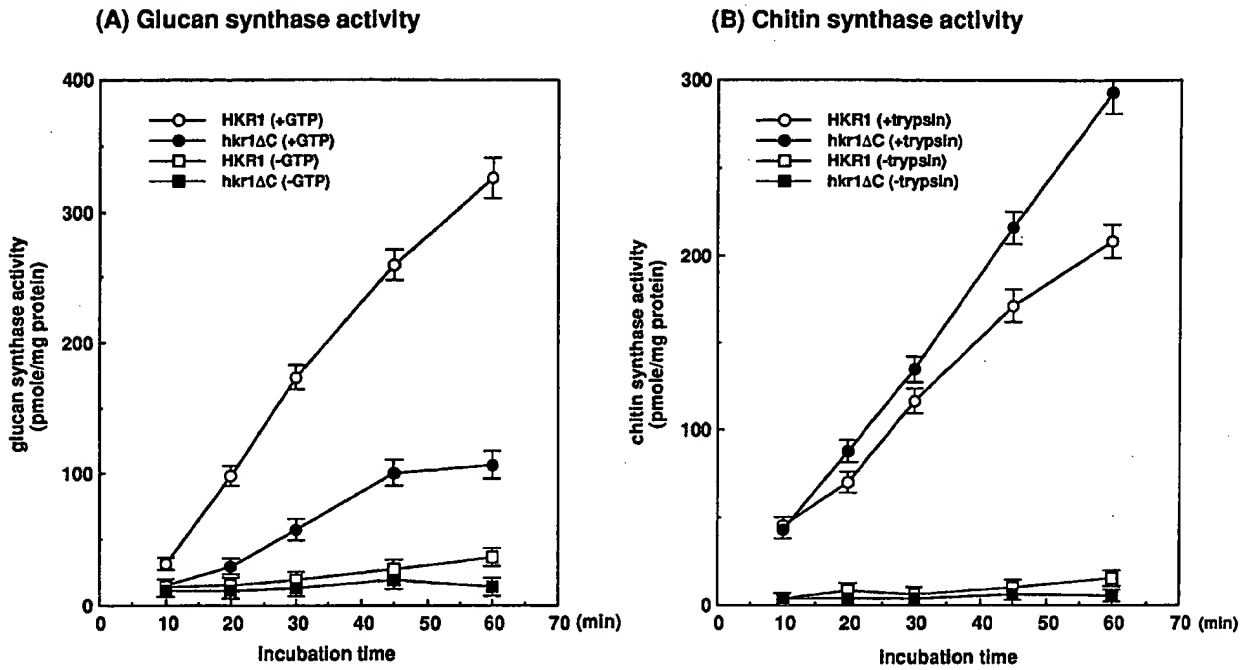

FIG. 2. Activities of β-1,3-glucan synthase and chitin synthase in HKR1 and hkr1ΔC cells. (A) Portions (20 μg) of proteins from total membranes prepared from HKR1 cells (open symbols) or hkr1ΔC cells (closed symbols) were assayed for β-1,3-glucan synthase in the presence (circles) or absence (squares) of GTP. (B) Portions (20 μg) of proteins from total membranes prepared from HKR1 cells (open symbols) or hkr1ΔC cells (closed symbols) were assayed for chitin synthase with (circle) or without (square) trypsin treatment of the membranes. For more details, see Materials and Methods.

cells were washed with PBS, they were treated with anti-Hkr1p antibody and with FITC-conjugated protein A and then examined with a fluorescence microscope.

RESULTS

Overexpression of *HKR1* confers resistance to HM-1 killer toxin, which interferes with β-glucan synthesis in vivo (38). Since the protein specified by this gene contains the Ca^{2+}-binding EF hand motif in the cytoplasmic domain (22) and the addition of EDTA severely inhibited the action of HM-1 killer toxin (data not shown), the EF hand motif was expected to be important for the function of Hkr1p. To address this possibility, we have generated the mutant yeast strain in which *HKR1* was partially disrupted by inserting the *LEU2* gene at the *Bam*HI site located just before the potential transmembrane domain of Hkr1p (Fig. 1A). Southern blotting revealed that the resulting strain, designated hkr1ΔC, harbored only the partially disrupted *HKR1* allele (Fig. 1B) and thereby would express the truncated Hkr1p that lacks the C-terminal cytoplasmic domain, including the EF hand motif. Although the deletion of nearly the whole region of *HKR1* (*Kpn*I-*Xba*I region) was lethal, neither growth nor sensitivity to HM-1 killer toxin was affected by the partial disruption of *HKR1* (data not

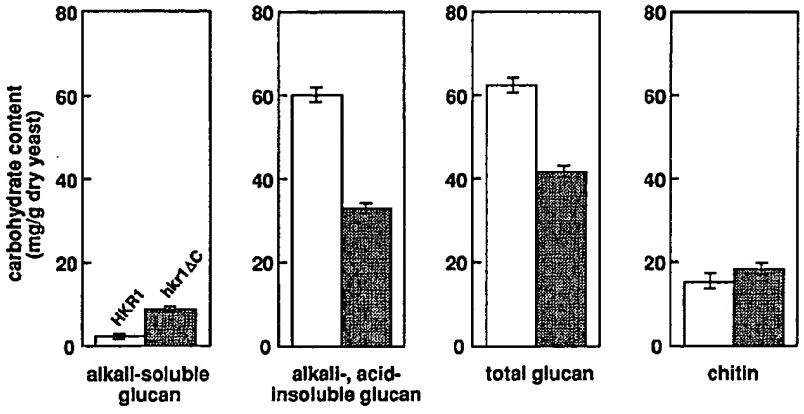

FIG. 3. Comparison of β-glucan and chitin levels in HKR1 and hkr1ΔC cells. HKR1 cells (white bars) or hkr1ΔC cells (shaded bars) in mid-logarithmic phase were harvested, and the levels of alkali-soluble, alkali- and acid-insoluble, and total glucan and chitin were analyzed. Glucan and chitin levels are given in milligrams per gram (dry weight) of yeast cells. For more details, see Materials and Methods.

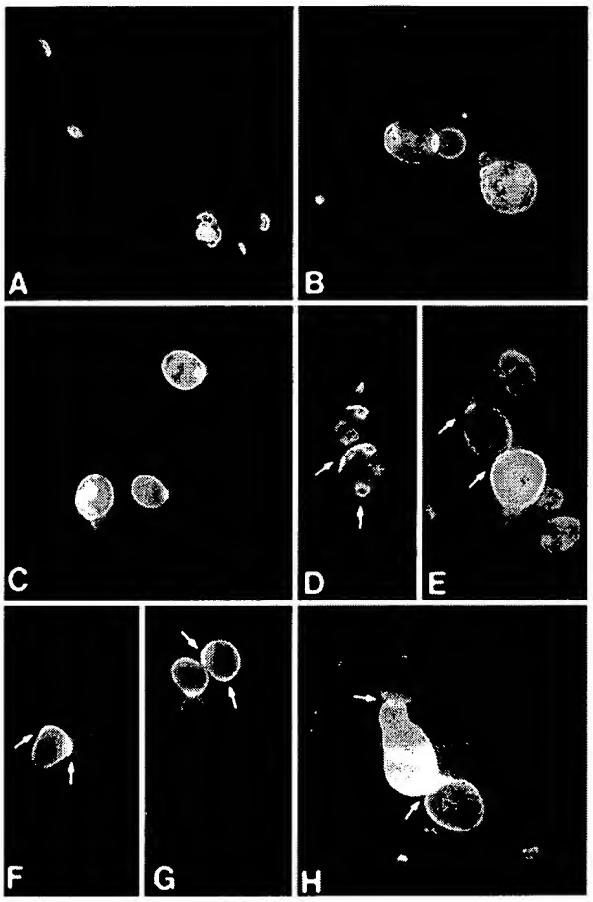

FIG. 4. Calcofluor White staining of HKR1 and hkr1ΔC cells. Haploid HKR1 (A), diploid RAY3A-D (B), or hkr1ΔC (C to H) cells in mid-logarithmic phase were harvested and stained with Calcofluor White. Typical patterns of bud scar staining for class I (C), class II (D and E), class III (F and G), and class III with abnormal morphology (H) seen in hkr1ΔC cells are shown. Strong fluorescence detected in bud scars of hkr1ΔC cells showing class II and class III staining patterns is indicated by arrows. For more details, see Materials and Methods.

shown), indicating that the C-terminal cytoplasmic domain of Hkr1p is not required for the essentiality and resistance to HM-1 toxin. It should be noted, however, that some cells of hkr1ΔC became swollen and were larger than those with the intact *HKR1* allele (see Fig. 4 and 5).

We previously demonstrated that the amount of β-glucan in the cell wall was increased in cells overexpressing *HKR1* and suggested that *HKR1* might regulate β-glucan synthesis in vivo. Therefore, we examined the effect of the partial disruption of *HKR1* on β-glucan synthesis. As shown in Fig. 2, GTP-dependent β-1,3-glucan synthase activity in hkr1ΔC cells was about one-third that of HKR1 cells, while trypsin-dependent chitin synthase activity was slightly increased in hkr1ΔC cells. Consistent with this result, the amounts of alkali- and acid-insoluble β-1,3-glucan content and total glucan were reduced to about 66% in hkr1ΔC cells, and the amount of chitin was not significantly different in these two strains (Fig. 3). All these results clearly indicate that *HKR1* is involved in β-1,3-glucan synthesis. The amount of alkali-soluble β-glucan (mixture of β-1,3- and β-1,6-glucan) was higher in hkr1ΔC cells, but this difference was very small compared with that of alkali- and acid-insoluble β-glucan.

Although *HKR1* did not rescue *cdc24* at nonpermissive temperature, *HKR1* has significant sequence homology with *MSB2*, a multicopy suppresser of *cdc24*, at both nucleotide and amino acid levels (33% identity and 45% homology at the amino acid level) (2, 22). *CDC24* is a gene required for the initiation of budding (34, 35), and Cdc24p catalyzes the release of guanine nucleotide (GDP) bound to Cdc42p to convert it into an active form (39). These facts prompted us to examine whether *HKR1* is also involved in the budding of yeast cells. Calcofluor White staining allowed us to divide yeast cells into three categories. Cells showing bud scars on one side, both sides, and several areas of the cells were classified as class I, class II, and class III, respectively. Haploid HKR1 cells and diploid RAY3A-D cells showed the typical pattern of axial budding and bipolar budding, respectively; when about 200 cells were examined, all HKR1 cells belonged to class I, while most RAY3A-D cells exhibited class II staining pattern (Fig. 4A and B and Table 1). However, hkr1ΔC cells budded in several ways. About half of the cells belonged to class I (Fig. 4C), and the rest of the cells were detected as class II (Fig. 4D and E) or class III (Fig. 4F to H). In addition, two-thirds of hkr1ΔC cells that belonged to class III showed an irregular form as shown in Fig. 4H, indicating that the axial budding pattern of haploid cells was not sustained in hkr1ΔC cells. The bud scar staining pattern of hkr1ΔC cells is also summarized in Table 1. The altered budding pattern of hkr1ΔC cells was further confirmed by scanning electron microscopy. While budding always occurred near the previous budding site that was revealed by the bud scars in the control haploid HKR1 cells (Fig. 5A), many of the hkr1ΔC cells budded in several directions and had bud scars in several areas (Fig. 5B and C).

As mentioned earlier, on the basis of its deduced amino acid sequence, Hkr1p is expected to be a type 1 membrane protein. We also addressed the subcellular localization of Hkr1p. For this purpose, a polyclonal antibody against Hkr1p was generated by immunizing rabbits with the bacterially expressed C-terminal portion of Hkr1p (from amino acid positions 1084 to

TABLE 1. Staining patterns of bud scars in RAY3A-D, HKR1, and hkr1ΔC cells[a]

Strain	No. of cells with staining pattern				Total no. of cells observed
	Class I	Class II	Class III	Class III deformed	
RAY3A-D (diploid)	37	161	0	0	198
HKR1 (haploid)	201	0	0	0	201
hkr1ΔC (haploid)	96	25	27	46	194

[a] RAY3A-D, HKR1, and hkr1ΔC cells were stained with Calcofluor White and divided into three classes on the basis of the pattern of staining. Cells showing bud scars on one side, both sides, and several areas of the cells are classified into class I, II, and III, respectively.

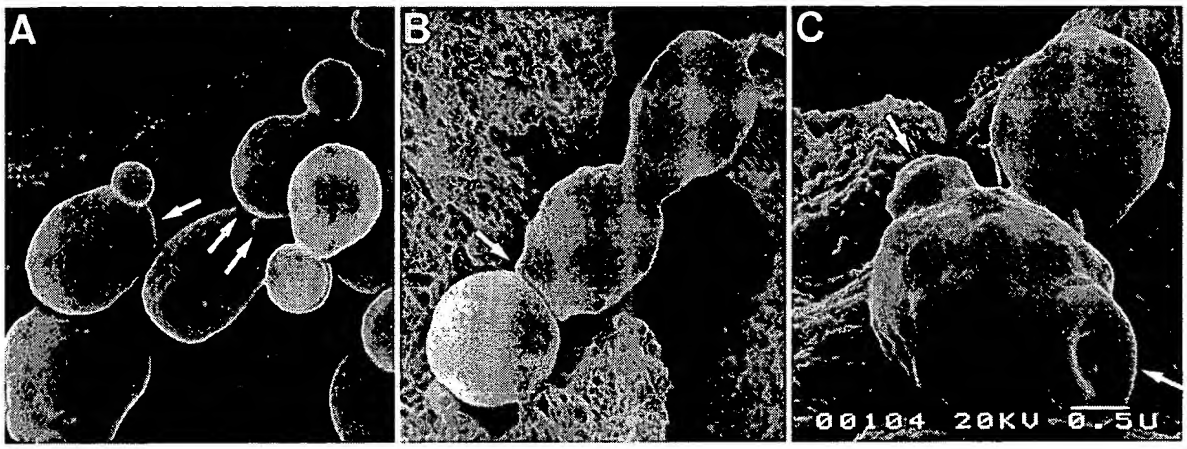

FIG. 5. Scanning electron microscopy of HKR1 and hkr1ΔC cells. HKR1 cells (A) or hkr1ΔC cells (B and C) in mid-logarithmic phase were harvested and fixed for scanning electron microscopy. Visible bud scars are indicated by arrows. For more details, see Materials and Methods.

1803). Since Hkr1p was expressed as a fusion protein with GST, Hkr1p was separated from carrier GST by treatment with thrombin and purified by affinity column chromatography. Both the 27-kDa GST and 60-kDa GST-Hkr1p fusion protein were strongly expressed in bacterial cells after treatment with IPTG (Fig. 6A). Western blotting of the bacterial cell extracts with anti-Hkr1p antibody revealed that anti-Hkr1p antibody strongly reacted with GST-Hkr1p but not with GST alone (Fig. 6B). Yeast cell extracts were also analyzed by Western blotting using the same antibody. When the intact *HKR1* gene (indicated as *HKR1*) or the 2.6-kb *Hin*dIII-*Hin*dIII fragment of *HKR1* (indicated as *HKR1*^{tr}), which encodes only the C-terminal part of Hkr1p, was expressed under the control of the *GAL7* promoter, 250- and 110-kDa proteins were detected in cells overexpressing *HKR1* and *HKR1*^{tr}, respectively. In addition, expression of 250- and 110-kDa proteins were observed only when cells were cultured in medium containing galactose, and nothing was detected in vector-transfected control (indicated as pMT) even when they were cultured in galactose-containing medium (Fig. 7). All these data clearly demonstrate that the antibody generated against the C-terminal portion of Hkr1p was highly specific to Hkr1p and that the endogenous Hkr1p was undetectable probably because of its low level of expression. The molecular mass of the protein detected by anti-Hkr1p antibody in cells overexpressing the intact *HKR1* gene was larger than that calculated from the deduced amino acid sequence (189 kDa). This difference may be due to the glycosylation of Hkr1p.

Then, the cells harboring intact *HKR1* whose transcription was under control of the *GAL7* promoter were stained with the anti-Hkr1p antibody described above together with FITC-conjugated protein A. When the cells were cultured in the medium containing galactose, strong fluorescence was observed in the cell surface in more than 90% of 1,000 cells examined (Fig.

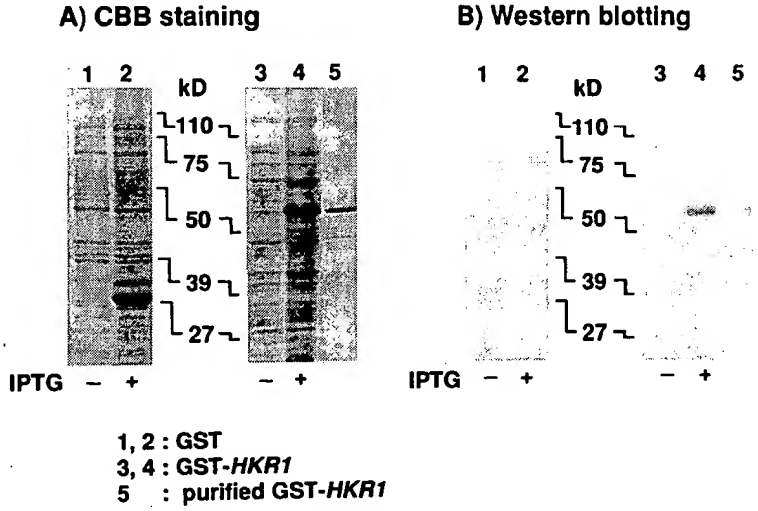

FIG. 6. Expression of Hkr1p in *E. coli* and generation of anti-Hkr1p antibody. About 30 μg of protein of crude *E. coli* extracts (lanes 1 to 4) or 1 μg of affinity-purified GST-Hkr1p fusion protein (lane 5) was fractionated on 10 or 12.5% polyacrylamide gels and stained with Coomassie brilliant blue (CBB) (A) or hybridized with anti-Hkr1p antibody (B). *E. coli* cells harboring pGEX2T (lanes 1 and 2) or pGEXHKR1 (lanes 3 and 4) were treated (+) or not treated (−) with IPTG before harvest. The positions (in kilodaltons) of protein size markers are indicated. For more details, see Materials and Methods.

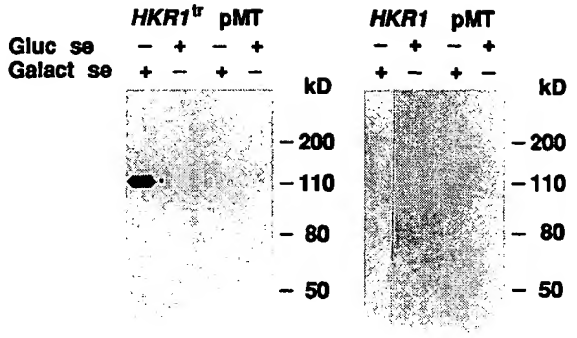

FIG. 7. Expression of Hkr1p in yeast cells. A451 cells harboring pMT34-317, pMTHKR1^{tr}, or pMTHKR1 are indicated over the lanes as pMT, *HKR1*^{tr}, or *HKR1*, respectively, and they were cultured in medium containing (+) glucose or galactose. Portions (50 μg) of proteins of whole-cell extracts were fractionated on SDS–7% polyacrylamide gels, and hybridized with anti-Hkr1p antibody. The positions (in kilodaltons) of protein size markers are indicated to the right of the gels. For more details, see Materials and Methods.

8A). In some cells, fluorescence was detected as patch-like spots in the periphery (Fig. 8B). In contrast, the whole cell was stained with anti-Hkr1p antibody when only the C-terminal half of Hkr1p was expressed (Fig. 8C and D), indicating that the N-terminal signal sequence was essential for the cell surface localization of Hkr1p. The fluorescence was specific to anti-Hkr1p antibody; treatment of the cells with control serum gave rise to only background level (Fig. 8F). We also stained the cells cultured in the glucose-containing medium with anti-Hkr1p antibody to see the localization of endogenous Hkr1p but failed to detect any significant fluorescence because of its very low level of expression (Fig. 8E). From these results, we concluded that Hkr1p is localized predominantly on the cell surface and regulated both β-glucan synthesis and bud site selection.

DISCUSSION

We have demonstrated that the partial disruption of *HKR1* that would eliminate the C-terminal cytoplasmic domain of Hkr1p did not affect cell growth but decreased β-1,3-glucan synthase activity and the level of β-1,3-glucan in the cell wall. Since the EF hand motif is located in the cytoplasmic domain of Hkr1p, the above result indicates that the EF hand motif of *HKR1* is not necessary for vegetative growth of yeast cells but is required for the normal level of cell wall β-1,3-glucan synthesis. Further, the finding that the partial disruption of *HKR1* altered the axial budding pattern of haploid cells strongly supports the idea that bud site selection of yeast cells is somehow related to the cell wall β-1,3-glucan synthesis.

A series of *BUD* genes is required for proper bud site selection. *BUD1*, *BUD2*, and *BUD5* are necessary for establishing both bipolar and axial budding patterns, and *BUD3* and *BUD4* play important roles in converting the budding pattern from bipolar to axial (11, 12). Since partial disruption of *HKR1* gave rise to mixed population of cells exhibiting different budding patterns, *HKR1* might affect the bud site selection not through *BUD* genes but by interacting with some other factors.

By indirect immunofluorescence microscopy, we have also demonstrated that Hkr1p is localized predominantly on the cell surface. The cell surface localization of Hkr1p is dependent on the N-terminal signal sequence because the C-terminal half of Hkr1p existed uniformly in the cells. Since β-1,3-glucan synthase is also thought to localize in membranes (15, 27, 33), one possible mechanism of control of β-1,3-glucan synthesis by *HKR1* is that Hkr1p is associated with β-1,3-glucan synthase and regulates its activity. However, our preliminary experiments did not support this hypothesis; Hkr1p was not coimmunoprecipitated with Fks1p or Gsc1p and was not copurified with β-1,3-glucan synthase activities. Thus, it seems likely that Hkr1p regulates cell wall β-1,3-glucan synthesis by an unknown mechanism that is independent of β-1,3-glucan synthase. In fact, overexpression of *HKR1* upregulates cell wall β-1,3-glucan content without affecting β-1,3-glucan synthase activity (22).

The level of full-length Hkr1p (250-kDa protein, indicated as *HKR1* in Fig. 7) expressed by the *GAL7* promoter was considerably lower than that of the C-terminal part of Hkr1p (110-kDa protein encoded by the 2.6-kb *Hin*dIII-*Hin*dIII fragment of *HKR1*, indicated as *HKR1*^{tr} in Fig. 7), even though they were expressed under the same promoter. Since the levels of mRNA of full-length *HKR1* and 2.6-kb *Hin*dIII-*Hin*dIII fragment of *HKR1* were almost the same (data not shown; also see reference 22), there seems to be a mechanism of regulation of Hkr1p expression at the posttranscriptional level. This posttranscriptional regulation of Hkr1p expression, if present, may also account for the low level of endogenous Hkr1p.

Interestingly, hkr1ΔC cells became less susceptible to Calcofluor White, which is believed to bind mainly to chitin and interferes with cell division in *S. cerevisiae* (29). However, the amount of cell wall chitin was not reduced in hkr1ΔC cells compared to that of control HKR1 cells (Fig. 3). According to the report of Kollar et al. (23), chitin synthase 3, which is a rare chitin synthase isozyme, is necessary for the formation of linkage between chitin and β-1,3-glucan. Therefore, we also examined whether chitin synthase 3 activity is affected by the partial disruption of *HKR1* by using nickel and cobalt ions (13) but did not find any significant difference between HKR1 and hkr1ΔC cells. Thus, the resistance of hkr1ΔC cells to Calcofluor White cannot simply be explained by the reduction of cell wall chitin content or defect in β-1,3-glucan-chitin linkage but may be due to changes in the ratio of cell wall polysaccharides. In fact, the amount of alkali-soluble β-glucan (mixture of β-1,3- and β-1,6-glucan) was higher in hkr1ΔC cells than in control cells, while

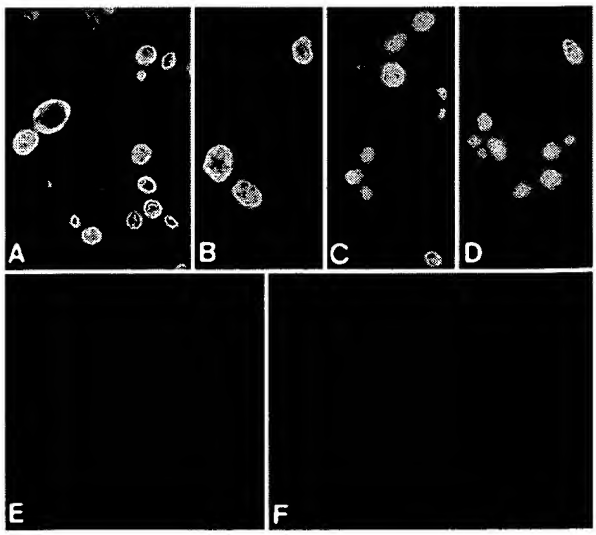

FIG. 8. Subcellular localization of Hkr1p. A451 cells harboring pMTHKR1 (A, B, E, and F) or pMTHKR1^{tr} (C and D) were cultured in medium containing galactose (A, B, C, D, and F) or glucose (E) and treated with the anti-Hkr1p antibody (A to E) or control rabbit immunoglobulin G (F) and then with FITC-conjugated protein A. For more details, see Materials and Methods.

the amounts of alkali- and acid-insoluble β-1,3-glucan were greatly reduced by the partial disruption of *HKR1* (Fig. 4).

We previously demonstrated that the replacement of *Kpn*I-*Xba*I region of *HKR1* with the *LEU2* gene was lethal and concluded that *HKR1* was an essential gene (22). In this study, we have shown that insertion of the *LEU2* gene at the *Bam*HI site of *HKR1* did not affect the viability of cells. This result indicates that the region required for the essentiality of *HKR1* is located further 5′ upstream of the *Bam*HI site of this gene. Experiments involving the insertion disruption of *HKR1* at various restriction endonuclease sites suggest that the region essential for the viability of yeast cells is located near or within the repetitive sequence found in the gene. Although the physiological function of the repetitive sequence in *HKR1* remains to be established, it would be of interest to examine whether the deletion of the repetitive sequence of *HKR1* alone leads to the lethal phenotype of yeast cells.

Hong et al. (20) isolated the *KNR4* gene that renders *S. cerevisiae* cells resistant to HM-1 killer toxin and reported that the expected product of *KNR4* was identical to Sml1p, a known nuclear protein. Although there is no significant sequence homology between *HKR1* and *KNR4*, disruption of *KNR4*, as in hkr1ΔC cells, leads to the decrease in β-1,3-glucan synthase activity and cell wall β-1,3-glucan content. Therefore, it is speculated that *KNR4* and *HKR1* function in the same pathway with regard to β-1,3-glucan synthesis.

ACKNOWLEDGMENTS

We thank E. Cabib for yeast strains and for the suggestions regarding the chitin synthase 3 assay.

This work was supported in part by grants in aid (glycotechnology program) from the Ministry of Agriculture, Forestry and Fisheries of Japan and from the Ministry of Science, Technology and Education of Japan to T.N.

REFERENCES

1. **Adams, A. E., D. I. Johnson, R. M. Longnecker, B. F. Sloat, and J. R. Pringle.** 1990. *CDC42* and *CDC43*, two additional genes involved in budding and establishment of cell polarity in the yeast *Saccharomyces cerevisiae*. J. Cell Biol. **111**:131–142.
2. **Bender, A., and J. R. Pringle.** 1992. A Ser/Thr rich multicopy suppression of a *cdc24* bud emergence defect. Yeast **8**:315–323.
3. **Boas, N. F.** 1953. Method for the determination of hexosamines in tissues. J. Biol. Chem. **204**:553–563.
4. **Boone, C., M. Goebl, R. Puccia, A.-M. Sdicu, and H. Bussey.** 1993. Yeast *KRE2* defines a new gene family encoding probable secretory proteins, and is required for the correct N-glycosylation of proteins. Genetics **130**:273–283.
5. **Boone, C., S. S. Sommer, A. Hensel, and H. Bussey.** 1990. Yeast *KRE* genes provide evidence for a pathway of cell wall β-glucan assembly. J. Cell Biol. **110**:1833–1843.
6. **Brown, J. L., and H. Bussey.** 1993. The yeast *KRE9* encodes an O glycoprotein involved in cell surface β-glucan assembly. Mol. Cell. Biol. **13**:6346–6356.
7. **Brown, J. L., Z. Kossaczka, B. Jiang, and H. Bussey.** 1993. A mutational analysis of killer toxin resistance in *Saccharomyces cerevisiae* identifies new genes involved in cell wall (1-6)-β-glucan synthesis. Genetics **133**:837–849.
8. **Brown, J. L., S. North, and H. Bussey.** 1993. *SKN7*, a yeast multicopy suppressor of a mutation affecting cell wall β-glucan assembly, encodes a product with domains homologous to prokaryotic two-component regulators and to heat shock transcription factors. J. Bacteriol. **175**:6908–6915.
9. **Cabib, E., and M. S. Kang.** 1987. Fungal 1,3-β-glucan synthase. Methods Enzymol. **138**:637–642.
10. **Cabib, E., M. S. Kang, and J. Au-Young.** 1987. Chitin synthase from *Saccharomyces cerevisiae*. Methods Enzymol. **138**:643–649.
11. **Chant, J., K. Corrado, J. R. Pringle, and I. Herskowitz.** 1991. Yeast *BUD5*, encoding a putative GDP-GTP exchange factor, is necessary for bud site selection and interacts with bud formation gene *BEM1*. Cell **65**:1213–1224.
12. **Chant, J., and I. Herskowitz.** 1991. Genetic control of bud site selection in yeast by a set of gene products that constitute a morphogenetic pathway. Cell **65**:1203–1212.
13. **Choi, W.-J., and E. Cabib.** 1994. The use of divalent cations and pH for the determination of specific yeast chitin synthetases. Anal. Biochem. **219**:368–372.
14. **Coligan, J. E., A. M. Kruisbeek, D. H. Maigulies, E. M. Shevach, and W. Strober.** 1991. Current protocols in immunology, p. 2.7.1-2.7.11. Wiley Interscience, New York.
15. **Douglas, C. M., F. Foor, J. A. Marrinan, N. Morin, J. B. Nielsen, A. M. Dahl, P. Mazur, W. Baginsky, W. Li, M. El-Sherbeini, J. A. Clemas, S. M. Mandala, B. R. Frommer, and M. B. Kurtz.** 1994. The *Saccharomyces cerevisiae FKS1* (*ETG1*) gene encodes an integral membrane protein which is a subunit of 1,3-β-D-glucan synthase. Proc. Natl. Acad. Sci. USA **91**:12907–12911.
16. **Douglas, C. M., J. A. Marrinan, W. Li, and M. B. Kurtz.** 1994. A *Saccharomyces cerevisiae* mutant with echinocandin-resistant 1,3-β-glucan synthase. J. Bacteriol. **176**:5686–5696.
17. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350–356.
18. **Fleet, G. H.** 1991. Cell walls, p. 199–277. *In* A. H. Rose and J. S. Harrinson (ed.), The yeasts, vol. 4. Academic Press, New York.
19. **Goldman, R. C., P. A. Sullivan, D. Zakula, and J. O. Capobianco.** 1995. Kinetics of β-1,3-glucan interaction at the donor and acceptor sites of the fungal glucosyltransferase encoded by the *BGL2* gene. Eur. J. Biochem. **227**:372–378.
20. **Hong, Z., P. Mann, N. H. Brown, L. E. Tran, K. J. Shaw, R. S. Hare, and B. Didomenico.** 1994. Cloning and characterization of *KNR4*, a yeast gene involved in (1,3)-β-glucan synthesis. Mol. Cell. Biol. **14**:1017–1025.
21. **Inoue, S. B., N. Takewaki, T. Takasuka, T. Mio, M. Adachi, Y. Fujii, C. Miyamoto, M. Arisawa, Y. Furuichi, and T. Watanabe.** 1995. Characterization and gene cloning of 1,3-D-glucan synthase from *Saccharomyces cerevisiae*. Eur. J. Biochem. **231**:845–854.
22. **Kasahara, S., H. Yamada, T. Mio, Y. Shiratori, C. Miyamoto, T. Yabe, T. Nakajima, E. Ichishima, and Y. Furuichi.** 1994. Cloning of the *Saccharomyces cerevisiae* gene whose overexpression overcomes the effects of HM-1 killer toxin, which inhibits β-glucan synthesis. J. Bacteriol. **176**:1488–1499.
23. **Kollar, R., E. Petrakova, G. Ashwell, P. W. Robbins, and E. Cabib.** 1995. Architecture of the yeast cell wall. The linkage between chitin and β(1-3)-glucan. J. Biol. Chem. **270**:1170–1178.
24. **Lundblad, V.** 1992. *Saccharomyces cerevisiae*, p. 13.11.1-13.11.5. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. Wiley Interscience, New York.
25. **Manners, D. J., A. J. Mason, and J. C. Patterson.** 1973. The structure of a beta-(1 leads to 3)-D-glucan from yeast cell walls. Biochem. J. **135**:19–30.
26. **Meaden, P., K. Hill, J. Wagner, D. Slipetz, S. S. Sommer, and H. Bussey.** 1990. The yeast *KRE5* gene encodes a probable endoplasmic reticulum protein required for (1→6)-β-D-glucan synthesis and normal cell growth. Mol. Cell. Biol. **10**:3013–3019.
27. **Mol, P. C., H.-M. Park, J. T. Mullins, and E. Cabib.** 1994. A GTP-binding protein regulates the activity of (1-3)-β-glucan synthase, an enzyme directly involved in yeast cell wall morphogenesis. J. Biol. Chem. **269**:31267–31274.
28. **Parker, C. G., L. I. Fessler, R. E. Nelson, and J. H. Fessler.** 1995. *Drosophila* UDP-glucose:glycoprotein glucosyltransferase sequence and characterization of an enzyme that distinguishes between denatured and native proteins. EMBO J. **14**:1294–1303.
29. **Pringle, J. R., R. A. Preston, A. E. M. Adams, T. Stearns, D. G. Drubin, B. K. Haarer, and E. W. Jones.** 1989. Fluorescent microscopy in yeast. Methods Cell Biol. **31**:357–434.
30. **Roemer, T., and H. Bussey.** 1991. Yeast β-glucan synthesis: *KRE6* encodes a predicted type II membrane protein required for glucan synthesis *in vivo* and for glucan synthase activity *in vitro*. Proc. Natl. Acad. Sci. USA **88**:11295–11299.
31. **Roemer, T., S. Delaney, and H. Bussey.** 1993. *SKN1* and *KRE6* define a pair of functional homologs encoding putative membrane proteins involved in β-glucan synthesis. Mol. Cell. Biol. **13**:4039–4048.
32. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Shematek, E. M., J. A. Braatz, and E. Cabib.** 1980. Biosynthesis of the yeast cell wall. Preparation and properties of β-(1,3) glucan synthase. J. Biol. Chem. **255**:888–894.
34. **Sloat, B. F., A. Adams, and J. R. Pringle.** 1981. Roles of the *CDC24* gene product in cellular morphogenesis during the *Saccharomyces cerevisiae* cell cycle. J. Cell Biol. **89**:395–405.
35. **Sloat, B. F., and J. R. Pringle.** 1978. A mutant of yeast defective in cellular morphogenesis. Science **200**:1171–1173.
36. **Smith, D. B., and K. S. Johnson.** 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione *S*-transferase. Gene **67**:31–40.
37. **Tajima, M., Y. Nogi, and T. Fukasawa.** 1986. Duplicate upstream activating sequences in the promoter region of the *Saccharomyces cerevisiae GAL7* gene. Mol. Cell. Biol. **6**:246–256.
38. **Yamamoto, T., T. Hiratani, H. Hirata, M. Imai, and H. Yamaguchi.** 1986. Killer toxin from *Hansenula mrakii* selectively inhibits cell wall synthesis in a sensitive yeast. FEBS Lett. **197**:50–54.
39. **Zheng, Y., R. Cerione, and A. Bender.** 1994. Control of the yeast bud-site assembly GTPase Cdc42. Catalysis of guanine nucleotide exchange by Cdc24 and stimulation of GTPase activity by Bem3. J. Biol. Chem. **269**:2369–2372.

STIC-ILL NPL

From: Lacourciere, Karen
Sent: Friday, May 10, 2002 3:43 PM
To: STIC-ILL
Subject: ill order

Please provide the following references for use in examining 09/898,556

Mol. Cell. Biol. (1988), 8(8), 3104-13

Kagaku to Seibutsu (1995), 33(3), 146-8

Fudan Xuebao, Ziran Kexueban (2000), 39(6), 692-694

JOURNAL OF BACTERIOLOGY, (1994 Mar) 176 (5) 1488-99.

JOURNAL OF BACTERIOLOGY, (1996 Jan) 178 (2) 477-83.

GENE, (1998 Nov 5) 222 (1) 61-7.

MOLECULAR AND GENERAL GENETICS, (1999 Mar) 261 (2) 275-80.

ANIMAL GENETICS, (2001 Dec) 32 (6) 375-9.

Thank-you!

Karen A. Lacourciere Ph.D.
CM1 11D09 GAU 1635
(703) 308-7523

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

ELSEVIER

Gene 222 (1998) 61–67

GENE
AN INTERNATIONAL JOURNAL ON GENES AND GENOMES

The Krüppel-type zinc finger family gene, HKR1, is induced in lung cancer by exposure to platinum drugs

Tetsuya Oguri [a], Osamu Katoh [b,*], Toshiaki Takahashi [a], Takeshi Isobe [a], Ken Kuramoto [c], Shitau Hirata [d], Michio Yamakido [a], Hiromitsu Watanabe [b]

[a] *Second Department of Internal Medicine, Hiroshima University School of Medicine, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8551, Japan*

[b] *Department of Environment and Mutation, Research Institute for Radiation Biology and Medicine, Hiroshima University, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8553, Japan*

[c] *Department of Hematology and Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8553, Japan*

[d] *Department of Otolaryngology, Hiroshima University School of Medicine, Kasumi 1-2-3, Minami-ku, Hiroshima, 734-8551, Japan*

Received 25 June 1998; received in revised form 28 August 1998; accepted 28 August 1998; Received by T. Sekiya

Abstract

To investigate the molecular mechanism associated with the signaling pathway of platinum drug administration, we focused on the C_2H_2-type zinc finger (ZNF) transcription factor gene family. Here we show cloning of a Krüppel-type ZNF gene, HKR1, which contains Krüppel-associated box (KRAB) domain and ZNF motifs. We found that mRNA expression of the HKR1 gene was induced in lung-cancer cell lines by exposure to cisplatin using Northern blot analysis. Moreover, we also found that HKR1 mRNA expression levels in lung cancers were higher than those in normal lung tissues, and that high expression levels in lung cancers were associated with antemortem platinum drug administration. These results suggest that HKR1 may be associated with the regulation of a signaling pathway involved in the progression of lung cancer or the acquisition of resistance to platinum drugs.

Keywords: Zinc finger motif; cDNA cloning; mRNA expression; Chemotherapy

1. Introduction

Since platinum drugs are some of the key drugs in lung-cancer chemotherapy, resistance to them is an important problem. Several mechanisms of resistance to platinum drugs, including increased levels of intracellular glutathione (Fujiwara et al., 1990) and glutathione-related enzymes (Godwin et al., 1992; Goto et al., 1995), and decreased intracellular drug accumulation associated with glutathione-S conjugate export pump activity (Ishikawa and Ali-Osman, 1993; Kurokawa et al., 1995), have been suggested. To examine the mechanism of platinum drug resistance in lung cancer, we previously studied the gene expression levels of the determinants in relation to platinum drug resistance (Oguri et al., 1998), and the results indicated that the acquisition of resistance to platinum drugs may be a result of the stress response of cancer cells. However, the molecular mechanisms of the signaling pathway of the stress response activated by exposure to platinum drugs are still uncertain.

To identify the transcriptional factors that mediate the signaling pathway activated by exposure to platinum drugs, we focused on a C_2H_2-type ZNF transcription factor gene family. It has been proposed that each motif is folded around a central zinc ion to form an independent minidomain, and that the most common role of the ZNF motif is to serve as DNA-binding domain within a transcription factor (Klug and Schwabe, 1995).

In this study, we screened the ZNF transcription factor genes induced in human lung-cancer cell lines by exposure to cisplatin using RT–PCR with degenerate

* Corresponding author. Tel: 81-82-257-5817; Fax: 81-82-256-7104; e-mail: katoho@mcai.med.hiroshima-u.ac.jp

Abbreviations: HKR1, Human Krüppel-related gene 1; KRAB, Krüppel-associated box; RT–PCR, reverse transcription polymerase chain reaction; ZNF, zinc finger.

0378-1119/98/$19.00
PII: S0378-1119(98)00464-8

primers, and we identified the HKR1 gene (Ruppert et al., 1988) as a candidate gene involved in the stress response. Furthermore, we cloned the region containing the KRAB domain and ZNF motifs of HKR1 cDNA and examined the gene expression in lung cancers in relation to platinum drug exposure.

2. Materials and methods

2.1. Cell lines and cell culture

A human lung adenocarcinoma cell line, PC-14 (Ohe et al., 1989), was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. For the induction experiments, parental cells in the late-log phase were harvested and seeded into tissue culture dishes at a density of 5×10^5 cells/ml. After a 1 h pre-incubation period, they were incubated with 5 μg/ml of cisplatin for 1, 4, 8 and 12 h. The cisplatin concentration and the duration of incubation were chosen as described previously (Fujiwara et al., 1990). Cisplatin was obtained from Bristol Myers Co., Tokyo.

2.2. Clinical samples

Twelve autopsy samples (six adenocarcinomas and six corresponding normal lung tissues) from six patients with lung cancer who were admitted to Hiroshima University Hospital were studied. Four of the six samples were obtained from untreated patients, and two of the six patients were treated only by carboplatin during life. Fresh specimens of primary lung tumors and normal lung tissues were obtained during autopsy after written informed consent had been obtained. The tissues were frozen in liquid nitrogen and stored at −80°C until they were analyzed.

2.3. Isolation of full-length HKR1 cDNA

The peptide sequences HTG(Q/H)KPF and CGRKFARS, which are conserved in the ZNF domain of EGR family, C_2H_2-type ZNF genes, were used to design degenerate primers for PCR-based cDNA cloning as described previously (Katoh et al., 1998). We used an upstream primer EG1 [5′-CA(CT)AC(ACGT)G G(ACGT)(GC)(AT)(ACGT)A(AG)(AG)CC(ACT)-TTCT) CA(AG)TG-3′] and a downstream primer EG2 [5′-GA(ACGT)C(CT)(ACGT)GC(AG)AA(CT)(C-T)T(CT)(CT)T(ACGT)(CG)(CG)(AG)CA-3′] to amplify ZNF genes from cDNA synthesized from the total RNA extracted from PC-14 cells that had been exposed to cisplatin. RT–PCR was carried out for 30 cycles using the EG1 and EG2 primers. The thermal cycle consisted of denaturation at 94°C for 30 s, annealing at 42°C for 3 min and extension at 72°C for 1 min. The PCR products were then electrophoresed on a 6% polyacrylamide gel, removed from the gel and subcloned into plasmid vectors.

To clone a full-length HKR1 cDNA, a cDNA library constructed from the poly(A)$^+$ RNA of a human leukemia cell line, CMK86 (Katoh et al., 1998), was screened using the PCR products derived from the HKR1 mRNA as the probes. This cDNA library was constructed using a cDNA synthesis kit (Amersham, UK) and a ZAP Express cloning kit (Stratagene, CA). The nucleotide sequence was determined by the dideoxy chain termination method after cloning the cDNA into the pBK-CMV phagemid vector following its in vivo excision from the ZAP Express vector.

2.4. Chromosome localization of the HKR1 gene by using radiation hybrid panels

The GeneBridge 4 Radiation Hybrid Panel (Research Genetics, Inc. Huntaville, AL) was used for chromosomal localization of the HKR1 gene. PCR was performed using a sense primer, 5′-CTCAGGAGACCA-GGAAAATGGC-3′ (154–176), and a reverse primer, 5′-TCCCCTCGCTCCAGCTGAGCAATG-3′ (357–380). The PCR conditions were denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min.

2.5. RNA extraction and Northern hybridization

Total cellular RNA was extracted from the cell lines or from the autopsy samples as described previously (Oguri et al., 1998). Northern blots of RNA extracted from the lung-cancer cell lines were hybridized with a probe specific for HKR1 (HKRUTR), which recognizes the 3′-untranslated region of the HKR1 cDNA (nucleotide number 2071–2781), as described previously (Fujiwara et al., 1990).

2.6. RT–PCR and quantification of PCR products

The cDNA derived from the RNA extracted from the autopsy samples was subjected to PCR amplification using the HKR1 gene primers described above. Then, PCR for the HKR1 cDNA was performed for 28 cycles under the conditions described above. The PCR products were electrophoresed, transferred to nylon membranes (Hybond N+; Amersham), and hybridized with ^{32}P-labeled cDNA probe as described previously (Oguri et al., 1998). The PCR products of the HKR1 gene described above were used as cDNA probes. We used β-actin gene as an internal control, and the sequence of PCR primers, the PCR procedure and a cDNA probe for β-actin gene were as described previously (Oguri et al., 1998). After washing, the radioactivity level was measured with a laser imaging analyzer (BAS-2000; Fuji

```
   1 GGCGCGTTAAGCTGGTTGGGACCCGGGAAGGCCTCCCTCTTAAGGTCTTTCCCACACCTCTGCTCCTTGTTACCTGACTTTCGGCTTCAG   90
   1 G A L S W L G P G K A S L L R S F P H L C S L L P D F R L Q                                  30
  91 GATCCGCGGCGTGCACCCGCGTTCCATCTGTCTTCTGAGACTTTGCCCTTCTCCAGGAAGAGCACTCAGGAGACCAGGAAAATGGCTACA  180
  31 D P R R A P A F H L S S E T L P F S R K S T Q E T R K (M) A T                                60
 181 GGGCTCCTGAGAGCCAAAAAAGAGGCGTTCGTGGCATTCAGGGATGTGGCTGTGTACTTCACCCAGGAGGAGTGGAGGTTGTTGAGCCCT  270
  61 G L L R A K K E A F V A F R D V A V Y F T Q E E W R L L S P                                  90
 271 GCTCAGAGGACCCTGCACAGGGAGGTGATGCTGGAGACTTATAACCATCTGGTCTCACTGGAAATTCCATCTTCTAAACCAAAACTCATT  360
  91 A Q R T L H R E V M L E T Y N H L V S L E I P S S K P K L I                                  120
 361 GCTCAGCTGGAGCGAGGGGAAGCGCCCTGGAGAGAGGAGAGAAAATGTCCACTGGACCTCTGTCCAGAATCGAAGCCAGAAATTCAACTT  450
 121 A Q L E R G E A P W R E E R K C P L D L C P E S K P E I Q L                                  150
 451 AGTCCCTCCTGCCCTCTGATTTTCTCCAGTCAGCAAGCTCTCAGCCAACATGTGTGGCTGAGTCATCTCTCTCAGCTGTTTTCAAGTTTA  540
 151 S P S C P L I F S S Q Q A L S Q H V W L S H L S Q L F S S L                                  180
 541 TGGGCAGGAAATCCTCTCCACCTGGGAAAACACTATCCAGAAGATCAGAAACAACAGCAGGATCCATTCTGCTTTAGTGGCAAAGCAGAA  630
 181 W A G N P L H L G K H Y P E D Q K Q Q Q D P F C F S G K A E                                  210
 631 TGGATTCAAGAGGGAGAAGACTCCAGACTCCTGTTTGGGAGAGTAAGCAAAAATGGCACTTCAAAGGCACTTTCCAGCCCACCTGAAGAA  720
 211 W I Q E G E D S R L L F G R V S K N G T S K A L S S P P E E                                  240
 721 CAACAGCCAGCACAGTCCAAGGAAGACAACACAGTGGTGGATATAGGGTCCAGCCCTGAACGGAGGGCAGATCTAGAGGAAACAGACAAA  810
 241 Q Q P A Q S K E D N T V V D I G S S P E R R A D L E E T D K                                  270
 811 GTATTGCATGGTTTAGAAGTCTCAGGATTTGGAGAAATCAAATATGAAGAGTTTGGGCCAGGCTTTATCAAGGAGTCAAACCTCCTTAGC  900
 271 V L H G L E V S G F G E I K Y E E F G P G F I K E S N L L S                                  300
 901 CTCCAGAAGACACAAACTGGGGAGACACCTTACATGTACACTGAGTGGGGAGACAGCTTTGGCAGTATGTCAGTCCTCATCAAAAACCCA  990
 301 L Q K T Q T G E T P Y M Y T E W G D S F G S M S V L I K N P                                  330
 991 AGGACACACTCTGGGGGAAAGCCTTATGTGTGCAGGGAATGTGGGCGAGGCTTTACGTGGAAGTCAAACCTGATCACACATCAGAGGACA 1080
 331 R T H S G G K P Y V C R E C G R G F T W K S N L I T H Q R T                                  360
1081 CACTCAGGGGAGAAACCTTATGTGTGCAAGGATTGTGGACGAGGCTTTACTTGGAAGTCGAACCTCTTTACACATCAGCGGACACACTCA 1170
 361 H S G E K P Y V C K D C G R G F T W K S N L F T H Q R T H S                                  390
1171 GGGCTCAAGCCTTATGTGTGCAAGGAATGTGGGCAGAGCTTTAGCCTGAAGTCAAACCTCATTACCCACCAGAGGGCGCACACTGGGGAG 1260
 391 G L K P Y V C K E C G Q S F S L K S N L I T H Q R A H T G E                                  420
1271 AAGCCTTATGTTTGCAGGGAATGTGGGCGTGGCTTTCGCCAGCATTCACACCTGGTCAGACACAAGAGGACACATTCAGGAGAGAAGCCT 1350
 421 K P Y V C R E C G R G F R Q H S H L V R H K R T H S G E K P                                  450
1351 TACATTTGCAGGGAGTGTGAGCAAGGCTTTAGCCAGAAGTCACACCTCATCAGACACTTAAGGACACACACAGGAGAGAAGCCTTATGTA 1440
 451 Y I C R E C E Q G F S Q K S H L I R H L R T H T G E K P Y V                                  480
1441 TGCACAGAATGTGGGCGTCACTTTAGCTGGAAATCAAACCTCAAAACACACCAGAGGACACACTCAGGGGTTAAACCTTATGTCTGCCTG 1530
 481 C T E C G R H F S W K S N L K T H Q R T H S G V K P Y V C L                                  510
1531 GAGTGCGGGCAGTGCTTTAGCCTGAAGTCAAACCTTAACAAACACCAGAGGTCACACACGGGGGAGAAGCCATTTGTATGTACGGAGTGT 1620
 511 E C G Q C F S L K S N L N K H Q R S H T G E K P F V C T E C                                  540
1621 GGGCGAGGCTTTACCCGGAAATCAACCCTGATCACGCACCAGAGGACACACTCAGGGGAGAAGCCATTTGTATGTGCTGAGTGTGGACGA 1710
 541 G R G F T R K S T L I T H Q R T H S G E K P F V C A E C G R                                  570
1711 GGCTTTAATGATAAGTCCACCCTCATTTCACACCAGAGGACACATTCAGGGGAAAAGCCTTTTATGTGCAGGGAGTGTGGCAGAAGGTTT 1800
 571 G F N D K S T L I S H Q R T H S G E K P F M C R E C G R R F                                  600
1801 CGGCAGAAGCCTAACCTGTTTAGGCACAAGAGGGCACACTCAGGTGCCTTTGTGTGCAGGGAGTGTGGGCAAGGCTTTTGTGCTAAGTTA 1890
 601 R Q K P N L F R H K R A H S G A F V C R E C G Q G F C A K L                                  630
1891 ACTCTCATTAAACACCAGAGAGCACACGCAGGGGGGAAGCCTCATGTGTGCAGGGAGTGTGGGCAAGGCTTTAGCCGGCAGTCACACCTC 1980
 631 T L I K H Q R A H A G G K P H V C R E C G Q G F S R Q S H L                                  660
1981 ATTAGACACCAGAGGACACATTCAGGAGAGAAGCCTTATATTTGCAGAAAGTGTGGACGGGGCTTTAGTCGGAAGTCCAACCTTATCAGA 2070
 661 I R H Q R T H S G E K P Y I C R K C G R G F S R K S N L I R                                  690
2071 CATCAGAGGACACACTCAGGATAGAAACTTTATGTGTATAGGGAATGTGGTACAGCCTTTAGCCAGGAGTCATACTTCATCAGACACCAG 2160
 691 H Q R T H S G *
2161 AGGACACACACAGTGCTGTGGCTTTTTCAGCCATTGCTAGATACCAAAGTGGAGACATTCTGTGTGTGATTATGCATGAGACTGTACTGG 2250

2251 TAAGACTTGTATCTCCATCCACCTGAAGGAGAATTGCTGGCTCATTTTCAGGAGCCCTGCCCTTCCTCACTGTGGATGGTGGGTTGTGGA 2340

2341 AACCCGGTCAGGTAATGATAGTGGCAGGAGGCAGTCAAATGCCCAGGCAGATAGGGGTGGGTACCTGGTGAAACCCAACCTTAAAGCTGA 2430

2431 AGACAGTCCCGGCTAAATCCTCATACTGAATTGAGAACCTGTCTTCCCATTTGGTGTGCTTTCCTCCGATTGATCCCAACCCTTCACCTA 2520

2521 TTTTACGTATACCTGCCCTTTCCTAATTGGTTTTTACACTGCTGTGCCCACCTTTTGAGTGGTGCCTTTGCATACTTACAAATCAGTCAA 2610

2611 CGTGTATTCCCCTATTCTGAGCCCATAAAAGACCCAGACTCAGCTGCAGTGAGGAGAGAAATCACCCTGCTGTGGAGGTTGGGGACCACT 2700

2701 CCCTGCATCCCCTCTCCACTGAGAGCTGTTCTTTTGCTC AATAAAATTCTTTTCTACCCATCCTCACCCTAAAAAAAAAAA 2781
```

Fig. 1. Nucleotide sequence of HKR1 cDNA and amino acid sequence of the deduced protein. A possible polyadenylation signal is underlined. A possible initiator methionine is marked by a open circle. The dashed line sequence was cloned previously (Ruppert et al., 1988). The cDNA sequence has been submitted to DDBJ/EMBL/GenBank DNA database with ENTRY ID 9805131805022239.

Photo Film, Tokyo). The radioactivity associated with gene expression in each sample was expressed as the yield of the HKR1 gene relative to that of the β-actin gene. Then the filters were exposed to X-ray film (Fuji Photo Film, Japan) at −80°C.

3. Results

3.1. Identification of HKR1 CDNA

To clone ZNF genes showing increased mRNA expression levels after exposure to platinum drugs, RT–PCR

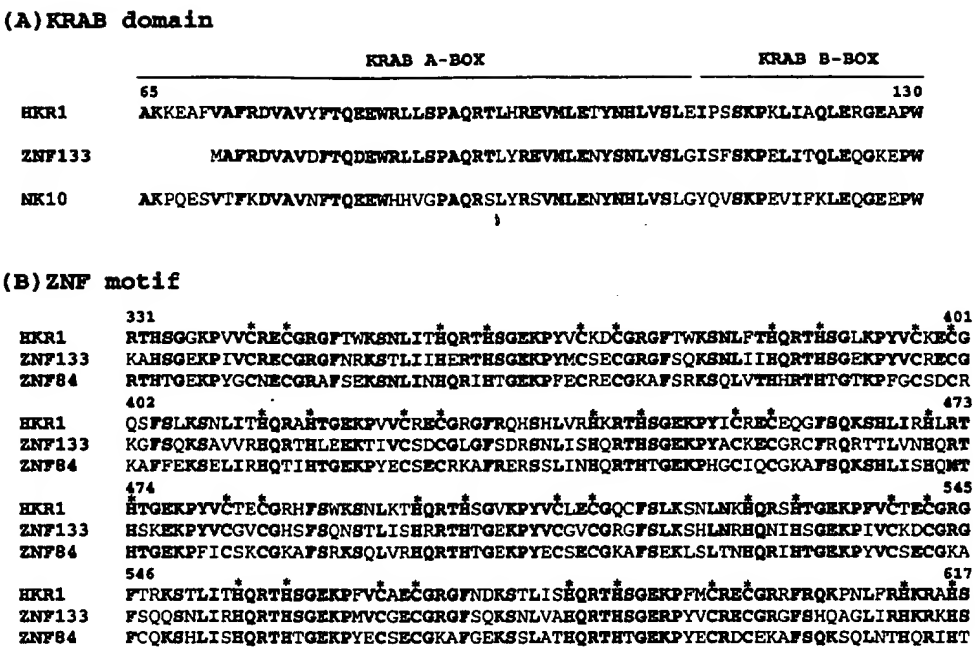

Fig. 2. Comparison of the (A) KRAB domain and the (B) ZNF motif of the HKR1 gene with those of other ZNF proteins. Bold letters indicate the amino acid residues identical to HKR1. Asterisks indicate the cystein and histidine residues conserved for ZNF motif.

was performed on the cDNA of PC-14 cells had been exposed to 5 μg/ml of cisplatin for 4 h, by using the degenerate primers EG1 and EG2. Several cDNA bands were detected by electrophoresis on a 6% polyacrylamide gel (data not shown). The PCR product in each band was eluted from the gel and subcloned into a plasmid vector. Northern blot hybridization analysis using the PCR products as probes revealed that the mRNA level of one clone, 85RH-2, was increased in PC-14 cells after exposure to cisplatin (data not shown). Nucleotide sequencing of 85RH-2 revealed that the clone was exactly identical to the human HKR1 gene (Ruppert et al., 1988). This finding suggested that 85RH-2 was a cDNA fragment derived from the HKR1 gene.

3.2. Cloning of a full-length HKR1 cDNA

To clone a full-length cDNA, approx. 5×10^5 plaques of a cDNA library derived from CMK86 cells was screened with the cDNA fragment of HKR1 as a probe. Three clones were isolated by the screening. Nucleotide sequencing of the longest clone revealed that the clone consisted of 2783 nucleotides, and that its deduced protein was composed of 697 amino acids (Fig. 1). We were unable to identify a definite initiator codon, but a candidate initiator methionine codon was located at codon 58 and was surrounded by a Kozak's consensus sequence (Kozak, 1991). This methionine site was followed by KRAB-A box and KRAB-B box. The protein had 13 consecutive repeated ZNF motifs at its C-terminus. The 120 N-terminus amino acids, including the KRAB domain, of the deduced protein showed high homology with human ZNF protein ZNF133 (73%) and with the mouse ZNF protein NK10 (62%) (Vissing et al., 1995; Lange et al., 1995). Furthermore, the 367 C-terminus amino acids, including the ZNF motifs, of the deduced protein showed high homology to human ZNF protein ZNF133 (64%) and ZNF84 (60%) (Vissing et al., 1995; Bellefroid et al., 1989; Fig. 2).

We determined the chromosomal location of HKR1 by using radiation hybrid panels. The database analysis showed that the HKR1 gene was very close to the chromosome maker D19S881 at chromosome 19q12-13.1 (data not shown).

3.3. Northern blot analysis

To confirm that HKR1 expression is induced by platinum drugs, we exposed PC-14 cells to cisplatin before Northern blot analysis, using a probe specific for HKR1. The results showed that the expression of HKR1 mRNA (3.0 kb) was induced by cisplatin in PC-14 cells (Fig. 3).

3.4. RT–PCR of lung-cancer cell lines and autopsy samples

First, we examined the PCR amplification using RNAs derived from all samples without reverse tran-

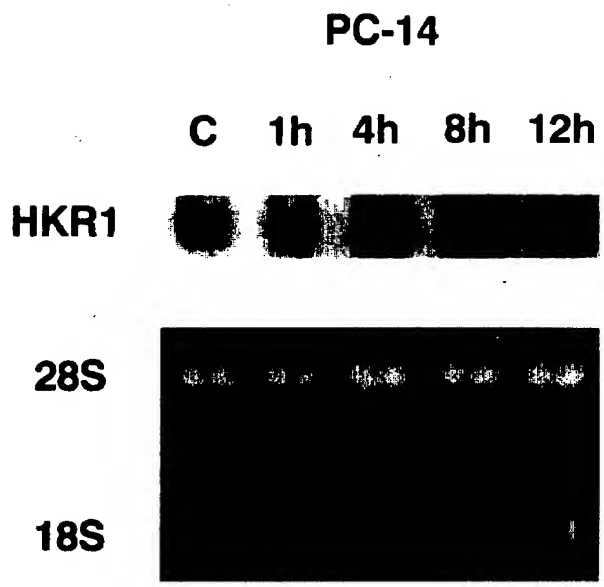

Fig. 3. Expression of HKR1 mRNA in PC-14 cells and their cells exposed to cisplatin by Northern blot analysis. 28S and 18S ribosomal RNAs stained by ethiduum bromide were used as an internal control. C: cells not exposed to cisplatin; 1h–12h: cells incubated with 5 μg/ml of cisplatin for 1, 4, 8 and 12 h.

scription reaction, the PCR products were not detected in all samples. Then, we examined HKR1 mRNA expression in PC-14 cells and in PC-14 cells exposed to cisplatin using quantitative RT–PCR. The PCR product of 227 bp derived from HKR1 mRNA was detected by Southern blot analysis. As shown in Fig. 4A, HKR1 mRNA expression was induced by platinum drugs.

Furthermore, we measured and compared the HKR1 expression levels in four lung adenocarcinomas and the corresponding normal lung tissues under the same RT–PCR condition. These tissues were not exposed to chemotherapeutic agents during life. The results show that HKR1 expression levels in lung cancers were higher than those in normal lung tissues (Fig. 4B). We also examined two lung adenocarcinomas from patients who were treated only with platinum drugs during life. The HKR1 expression levels in the platinum drug-exposed tumors increased approximately 3-fold compared to those in tumors not exposed to platinum drugs (Fig. 4B).

4. Discussion

In this study, we cloned the HKR1 gene cDNA, which we used to demonstrate that HKR1 gene expression levels were elevated in lung cancer specimens and that HKR1 expression is induced by platinum drugs.

We found that the HKR1 gene belongs to the Krüppel-type ZNF gene family, which contains both the KRAB-A and KRAB-B boxes. KRAB domain usually locates at the N-terminus of the Krüppel-type ZNF gene family. Therefore, a methionine that we observed at codon 57, upstream from a KRAB domain, might be a candidate initiator codon. Previously, it was reported that one-third of the Krüppel-type ZNF genes serve KRAB-A and KRAB-B boxes (Bellefroid et al., 1991), and that the KRAB domain mediates transcriptional repression (Witzgall et al., 1994, Margolin et al.,

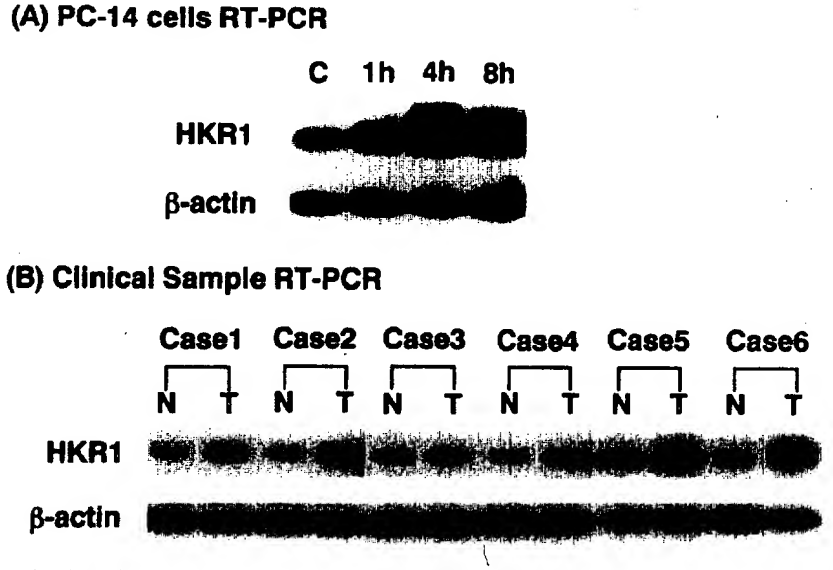

Fig. 4. HKR1 mRNA expression by RT–PCR. (A) PC-14 cells and PC-14 cells exposed to cisplatin. C: cells not exposed to cisplatin; 1h–8h: cells incubated with 5 μg/ml of cisplatin for 1, 4, 8 h. (B) Eight autopsy samples of four adenocarcinomas and four corresponding normal lung tissues from antemortem untreated patients (case 1–case 4), and four autopsy samples of two adenocarcinomas and two corresponding normal lung tissues from patients medicated only with carboplatin in life (cases 5 and 6). N: normal lung tissue; T: adenocarcinoma. β-actin was used as an internal control.

1994). However, the functions of the HKR1 remain to be elucidated, although ZNF133, which shows high homology to HKR1, was demonstrated to mediate transcriptional repression (Vissing et al., 1995).

The present study demonstrated that the HKR1 expression levels in lung-cancer tissues were higher than those in normal lung tissues. We confirmed that the HKR1 gene is located on chromosome 19q (Ruppert et al., 1988); an increased copy number of genes located at chromosome 19q was implicated in advanced ovarian cancer and advanced cervical cancer in comparative genomic hybridization studies (Arnold et al., 1996; Heselmeyer et al., 1997). Although we have not done Southern blot analyses on the DNA from our clinical samples because of obtaining small amounts of tissue for DNA extraction, these results indicate that HKR1 might be involved in oncogenesis or in the progression of cancers.

Furthermore, we demonstrated induction of HKR1 gene expression by platinum drugs in lung cancer, both in vitro and in vivo. Although the intervals between the last platinum drug dose and death of the patient were 4 and 5 months, it has been demonstrated that platinum persists in tissues from several months to years after the final dose (Tothill et al., 1992). This platinum persistence may induce chronic overexpression of HKR1 mRNA. We have found that expression of mRNA for γ-glutamylcysteine synthetase, the rate-limiting enzyme of glutathione synthesis, was associated with platinum drug exposure in the samples of examined in this study, and that γ-glutamylcysteine synthetase expression was induced by platinum drugs (Oguri et al., 1998). Although, it remains uncertain whether HKR1 is associated with the induction of mRNA by platinum drugs, and whether HKR1 is concerned with the resistance to or metabolism of platinum drugs by cancer cells, the HKR1 gene may mediate the stress response of lung-cancer cells to exposure to platinum drugs. Further studies are required to clarify the association between HKR1 mRNA overexpression and platinum drug resistance and/or metabolism.

Acknowledgement

This study was supported by Grants-in-Aid for Scientific Research (C) from the Ministry of Education, Science, Sports and Culture of Japan. We are grateful to Drs N. Saijo and K. Nishio (Pharmacology Division, National Cancer Center Research Institute) for providing the PC-14 cells. Thanks are also extended to Dr J. Yokota (Biology Division, National Cancer Center Research Institute) for his valuable suggestions.

References

Arnold, N., Hagele, L., Walz, L., Schempp, W., Pfisterer, J., Bauknecht, T., Kiechle, M., 1996. Overrepresentation of 3q and 8q material and loss of 18q material are recurrent findings in advanced human ovarian cancer. Genes Chromosomes Cancer 16, 46–54.

Bellefroid, E.J., Lecocq, P.J., Benhida, A., Poncelet, D.A., Belayew, A., Martial, J.A., 1989. The human genome contains hundreds of genes coding for finger proteins of the Krüppel type. DNA 8, 377–387.

Bellefroid, E.J., Poncelet, D.A., Lecocq, P.J., Revelant, O., Martial, J.A., 1991. The evolutionarily conserved Krüppel-associated box domain defines a subfamily of eukaryotic multifingered proteins. Proc. Natl. Acad. Sci. USA 88, 3608–3612.

Fujiwara, Y., Sugimoto, Y., Kasahara, K., Bungo, M., Yamakido, M., Tew, K.D., Saijo, N., 1990. Determinants of drug response in a cisplatin-resistant human lung cancer cell line. Jpn. J. Cancer Res. 81, 527–535.

Godwin, A.K., Meister, A., O'Dwyer, P.J., Huang, C.S., Hamilton, T.C., Anderson, M.E., 1992. High resistance to cisplatin in human ovarian cancer cell lines is associated with marked increase of glutathione synthesis. Proc. Natl. Acad. Sci. USA 89, 3070–3074.

Goto, S., Yoshida, K., Morikawa, T., Urata, Y., Suzuki, K., Kondo, T., 1995. Augmentation of transport for cisplatin-glutathione adduct in cisplatin-resistant cancer cells. Cancer Res. 55, 4297–4301.

Heselmeyer, K., Macville, M., Schröck, E., Blegen, H., Hellström, A.C., Shah, K., Auer, G., Reid, T., 1997. Advanced-stage cervical carcinomas are defined by a recurrent pattern of chromosomal aberrations revealing high genetic instability and a consistent gain of chromosome arm 3q. Genes Chromosomes Cancer 19, 233–240.

Ishikawa, T., Ali-Osman, F., 1993. Glutathione-associated *cis*-diamminedichloroplatinum(II) metabolism and ATP-dependent efflux from leukemia cells. J. Biol. Chem. 268, 20116–20125.

Katoh, O., Oguri, T., Takahashi, T., Takai, S., Fujiwara, Y., Watanabe, H., 1998. ZK1, a novel kruppel-type zinc finger gene, is induced following exposure to ionizing radiation and enhances apoptotic cell death on hematopoietic cells. Biochem. Biophys. Res. Commun. 249, 595–600.

Klug, A., Schwabe, J.W.R., 1995. Zing fingers. FASEB J. 9, 597–604.

Kozak, M., 1991. An analysis of vertebrate mRNA sequence: intimations of translational control. J. Cell Biol. 115, 887–903.

Kurokawa, H., Ishida, T., Nishio, K., Arioka, H., Sata, M., Fukumoto, H., Miura, M., Saijo, N., 1995. γ-Glutamylcysteine synthetase gene overexpression results in increased activity of the ATP-dependent glutathione S-conjugate export pump and cisplatin resistance. Biochem. Biophys. Res. Commun. 216, 258–264.

Lange, R., Christoph, A., Thiesen, H.J., Vopper, G., Johnson, K.R., Lemaire, L., Plomann, M., Cremer, H., Barthels, D., Heinlein, U.A.O., 1995. Developmentally regulated mouse gene NK10 encodes a zinc finger repressor protein with differential DNA-binding domains. DNA Cell Biol. 14, 971–981.

Margolin, J.F., Friedman, J.R., Meyer, W.K.-H., Vissing, H., Thiesen, H.-J., Rauscher III, F.J., 1994. Kruppel-associated boxes are potent transcriptional repression domains. Proc. Natl. Acad. Sci. USA 91, 4509–4513.

Oguri, T., Fujiwara, Y., Isobe, T., Katoh, O., Watanabe, H., Yamakido, M., Expression of γ-glutamylcysteine synthetase (γ-GCS) and multidrug resistance-associated protein (MRP), but not human canalicular multispecific organic anion transporter (cMOAT), genes correlates with exposure of human lung cancers to platinum drugs. 1998. Br. J. Cancer 77, 1089–1096.

Ohe, Y., Nakagawa, K., Fujiwara, Y., Sasaki, Y., Minato, K., Bungo, M., Niimi, S., Horichi, N., Fukuda, M., Saijo, N., 1989. In vitro evaluation of the new anticancer agents KT6149, MX-2, SM5887, menogarii, and liblomycin using cisplatin- or adriamycin-resistant human cancer cell lines. Cancer Res. 49, 4098–4102.

Ruppert, J., Kinzler, K.W., Wong, A.J., Bigner, S.H., Kao, F.-T., Law, M.L., Seuanez, H.N., O'Brien, S.J., Vogelstein, B., 1988. The GLI-Kruppel family of human genes. Mol. Cell Biol. 8, 3104–3113.

Tothill, P., Klys, H.S., Matheson, L.M., McKay, K., Smyth, J.F., 1992. The long-term retention of platinum in human tissues following the administration of cisplatin or carboplatin for cancer. Eur. J. Cancer 28, 1358–1361.

Vissing, H., Meyer, W.K., Aagaard, L., Tommerup, N., Thiesen, H.-J., 1995. Repression of transcriptional activity by heterologous KRAB domains present in zinc finger proteins. FEBS Lett. 369, 153–157.

Witzgall, R., O'Leary, E., Leaf, A., Önaldi, D., Bonventre, J.V., 1994. The Krüppel-associated box-A (KRAB-A) domain of zinc finger proteins mediates transcriptional repression. Proc. Natl. Acad. Sci. USA 91, 4514–4518.

STIC-ILL

From: Lacourciere, Karen
Sent: Friday, May 10, 2002 3:43 PM
To: STIC-ILL
Subject: ill order

Please provide the following references for use in examining 09/898,556

Mol. Cell. Biol. (1988), 8(8), 3104-13

Kagaku to Seibutsu (1995), 33(3), 146-8

Fudan Xuebao, Ziran Kexueban (2000), 39(6), 692-694

JOURNAL OF BACTERIOLOGY, (1994 Mar) 176 (5) 1488-99.

JOURNAL OF BACTERIOLOGY, (1996 Jan) 178 (2) 477-83.

GENE, (1998 Nov 5) 222 (1) 61-7.

MOLECULAR AND GENERAL GENETICS, (1999 Mar) 261 (2) 275-80.

ANIMAL GENETICS, (2001 Dec) 32 (6) 375-9.

Thank-you!

Karen A. Lacourciere Ph.D.
CM1 11D09 GAU 1635
(703) 308-7523

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

Mol Gen Genet (1999) 261: 275–280

ORIGINAL PAPER

L. D. Polley

Genetic analysis of mutant clones of *Chlamydomonas reinhardtii* defective in potassium transport

Received: 24 August 1998 / Accepted: 16 November 1998

Abstract Mutant clones of *Chlamydomonas reinhardtii* defective for potassium transport were isolated and characterized. Of the four genes identified, three – *TRK1*, *TRK2* and *TRK3* – encode high-affinity transport functions, and one gene, *HKR1*, encodes a low-affinity transport function. Characterization of the potassium dependence of recombinants possessing two mutant *trk* alleles suggests that the protein products of *TRK2* and *TRK3* interact functionally, and that *TRK1* may serve a regulatory function. The mutant clone defective for a low-affinity potassium transporter was isolated by mutagenizing *trk2-1* cells, which lack a functional high-affinity transporter, and screening surviving cells for dependence on very high potassium concentrations. The *hkr1* phenotype is expressed only in the presence of *trk2-1*.

Key words *Chlamydomonas reinrardtii* · Potassium transport · Membrane transport · Transport mutant

Introduction

Potassium is the major monovalent cation in plant and algal cells, and it is essential for a number of fundamental cellular processes, including protein synthesis, osmoregulation and regulation of membrane potential (Leigh and Wyn-Jones 1984). Given its importance to cell function, it is not surprising that the transport of potassium is highly regulated. It is generally accepted that there are at least two transport systems which operate in parallel in the plasma membrane (Epstein 1973). These systems have been defined kinetically as high-affinity transport and low-affinity transport. The high-affinity system exhibits typical Michaelis-Menten transport kinetics, but the kinetic behavior of the low-affinity system has been regarded as non-saturating (Kochian and Lucas 1988). The interplay of these systems is thought to provide the plant cell, which may encounter a wide range of environmental potassium concentrations, with the regulatory flexibility necessary to maintain homeostatic concentrations of cellular potassium (Maathuis and Sanders 1997).

In recent years, molecular and electrophysiological studies have provided considerable insight into the mechanisms and regulation of potassium transport. cDNA clones for both low-affinity (Anderson et al. 1992; Sentenac et al. 1992) and high-affinity (Schachtman and Schroeder 1994; Kim et al. 1998; Fu and Luan 1998) potassium transporters have been isolated. Additional molecular studies indicate that these cDNA clones are members of multi-gene families and that the expression of these genes is modulated (Cao et al. 1995; Kim et al. 1998). Patch clamp studies have been done on root hair protoplasts and on frog oocytes expressing plant potassium transport proteins. These studies have demonstrated that the low-affinity transporter is an inward rectifying K^+ channel (Schachtman et al. 1992; Gassman and Schroeder 1994) and that the high-affinity transporters operate either as a K^+/H^+ or K^+/Na^+ symporters (Maathuis and Sanders 1994; Rubio et al. 1995).

Despite the remarkable progress in our understanding of potassium transport in plants, there may be limits to the information that current molecular approaches can provide. Specifically, genes that regulate expression of potassium transporters or genes that encode heteromeric potassium transporters would be missed in screens that employ cDNA complementation of potassium transport-defective yeast. The use of *Chlamydomonas reinhardtii* to study potassium transport, however, may provide a means for overcoming the limitations of current molecular studies, as well as providing additional comparative data that should complement studies in higher plant systems. *Chlamydomonas*, like higher plants, has been reported to exhibit biphasic kinetics of uptake, and

Communicated by H. Saedler

L. D. Polley
Wabash College, Department of Biology
Crawfordsville, IN 47933, USA
Tel.: +1-765-3616219, Fax: +1-765-3616295

the lower-affinity system appears to be non-saturating (Malhorta and Glass 1995). However, this single-celled green alga represents a simpler experimental system, in that it lacks the various tissues and intergrated organ systems present in higher plants. Perhaps its most significant feature is the ability to isolate mutant clones defective for transport, which should make possible the identification and cloning of genes that would be missed by cDNA complementation of yeast or bacterial transport-defective mutants. The genetic analysis of potassium transport-defective mutant clones isolated previously (Polley and Doctor 1985) and for this study, are reported here. The data obtained from these analyses support the conclusion that at least two transport systems, a high-affinity and a low-affinity system, operate in *Chlamydomonas*. The high-affinity system is encoded by at least two genes, and the mutation in one clone, KDP4, is associated with a phenotype consistent with altered regulation of potassium transport.

Materials and methods

Strains

All strains used in this study were obtained from the *Chlamydomonas* Genetics Center (Duke University, Durham, N.C.). The original potassium transport-deficient mutant clones KDP1, KDP2 and KDP3 were obtained by UV mutagenesis of the wild-type strain R3 + NM (Polley and Doctor 1985). The mutant alleles *trk2–2* and *trk3* in clones KDP2 and KDP3, respectively, were subsequently rescued from meiotic lethal mutations in R3 + NM by crossing mutant strains to the wild-type strain 137c (*Chlamydomonas* Genetics Center strain CC125) and subsequently back crossing survivors with the *trk* phenotype to CC125. These back crosses were carried out for seven generations. Following the same UV mutagenesis protocol, the transport-deficient clones KDP4 (*trk1*) and KDP5 (*trk2-1*) were isolated from strain CC125. Strain DP2 (*trk2-1 act1 mt-*) is a recombinant from a cross between KDP5 (*trk2-1 mt+*) and CC1680 (*act1 ac80 mt-*). Mutant clones 10KDP1 and 50KDP1 were isolated after UV mutagenesis of DP2.

Media and growth conditions

Cells were grown axenically at 25° C under continuous illumination either in aerated liquid cultures or on medium solidified with agar. Medium 0K0N is a modification of TRIS-acetate-phosphate medium (TAP medium) that has only trace amounts of potassium and sodium (Polley and Doctor 1985). The potassium requirement of mutant clones was determined by measuring the rate of growth in 0K0 N supplemented with different concentrations of KCl. Cell growth was monitored by absorbance at 560 nm. Absorbance is directly proportional to cell number, and the same correlation between cell number and absorbance exists for cells grown in media containing different concentrations of potassium.

Mutagenesis and genetic analysis

Four milliliters of DP2 cells grown to a density of approximately 5×10^6 cells/ml were placed in a 4.5 cm petri dish and exposed to UV irradiation for 90 s in a Stratagene UV Crosslinker. After exposure, cells were grown overnight in the dark to fix the mutation. The cells were then plated on either 10K0N or 50K0N (0K0N medium supplemented with 10 or 50 mM KCl, respectively). Approximately 5–10% of the cells survive UV irradiation, and these were screened by replica-plating either to 1K0N (0K0N supplemented with 1 mM KCl) or to 0K0N solidified with purified agar (Difco Laboratories, Detroit, Mich., or BBL Becton Dickinson Microbiological Systems, Cockeysville, Md.). Mating, tetrad analysis and complementation were done as described by Harris (1989).

Potassium transport studies

Potassium transport by wild-type and mutant clones was characterized by following the uptake of ^{86}Rb as previously described (Polley and Doctor 1985). ^{86}Rb was employed because its half-life is significantly longer than that of ^{42}K (18.8 days versus 12 h). Cells grown to a density of $2–4 \times 10^6$ cells/ml were incubated in 0K0N containing 0.33 μCi ^{86}Rb and different concentrations of RbCl. Cells were collected on Gelman 0.8 μM cellulose acetate filters after a 5-min incubation, and then washed three times with unlabeled 0K0N. The amount of label taken up by the cells was determined by scintillation spectrometry.

Results

Analysis of a high-affinity potassium transporter

Wild-type cells of *Chlamydomonas* grow equally well in the presence of potassium concentrations ranging from 50 μM to 5.0 mM. Potassium transport-deficient mutant clones were initially isolated by their dependence on potassium concentrations greater than 0.1 mM; at concentrations of 0.1 mM potassium or less, these cells grow poorly (Polley and Doctor 1985). Since that initial report, two additional mutant clones, KDP4 and KDP5, were isolated by the same procedure. Their growth rates as a function of potassium concentration are shown in Table 1 and are compared to those of the wild type and KDP3r, a mutant clone derived from the rescue of the original KDP3 clone. This dependence on greater concentrations of potassium to sustain wild-type growth rates was shown to correlate with altered kinetics of potassium transport (Polley and Doctor 1985; see Table 4).

The potassium-dependent clones were characterized genetically by complementation (Table 2) and by tetrad analyses (Table 3). The complementation data demonstrate that there are at least three *trk* (*tr*ansport of *K*) genes and that they are completely recessive to their wild-type alleles. Tetrad analysis of various crosses shows that the *trk* loci are unlinked: *trk1* is on linkage group X, *trk2* is on linkage group II between *pf18* and *nic2*, and *trk3* is closely linked to *pf15* on linkage group III.

In an effort to acquire additional insight into the functional relationship between the *trk* alleles, double mutant recombinants were constructed, and their growth rates were determined as a function of potassium concentration (Table 1). The growth rates of clones KDP3r, KDP5, and DP7 at the different potassium concentrations are very similar; this indicates that the protein products of *TRK2-1* and *TRK3* probably interact functionally. Clone KDP4, by contrast, exhibits a hypomorphic or "leaky" potassium-dependent phenotype. While it grows at a slightly reduced rate in 1.0 and 0.5 mM KCl, it grows at a higher rate in 0.05 mM KCl

Table 1 Growth rates of wild-type and mutant clones at different potassium concentrations

Clone	Allele	Growth rates[a]				
		5.0 mM KCl	1.0 mM KCl	0.5 mM KCl	0.1 mM KCl	0.05 mM KCl
CC125	*TRK*	7.5±0.5	7.7±0.9	8.1±1.1	7.9±1.0	7.3±2.0
KDP3r	*trk3*	7.5±0.6	7.4±0.3	7.3±0.8	2.5±1.8	0.5±0.5
KDP4	*trk1*	6.1±0.6	4.7±0.7	3.9±0.8	2.6±0.9	1.8±0.8
KDP5	*trk2-1*	7.5±0.5	6.9±1.0	6.4±1.1	3.4±2.3	0.3±0.5
DP3	*trk1 trk2-1*		3.1±0.6	2.6±1.0	0.7±1.0	0.5±0.8
DP5	*trk1 trk3*		4.4±0.3	3.9±0.3	0.0	0.0
DP7	*trk2-1 trk3*		6.4±0.5	5.7±0.8	2.6±0.7	0.5±0.7

[a] Growth rates are expressed in terms of the growth rate constant $k \times 10^{-2}$. The constant k is determined from the expression $A_t = A_0 e^{kt}$, where A is the absorbance at 560 nm and t the time in h. A growth rate constant of 7.5×10^{-2} is equivalent to a doubling time of about 9 h; a growth rate constant of 2.5×10^{-2} gives a doubling time of about 28 h. The values shown are the means and standard errors of three experiments

than does either KDP3r or KDP5. Recombinant clones DP3 and DP5 exhibit similar growth rates at different potassium concentrations, and the overall phenotype for each is a combination of those seen with each mutant allele, i.e. *trk1* + *trk2-1* (DP3) and *trk1* + *trk3* (DP5). This suggests that the protein product of *TRK1* does not interact directly with the product of *TRK2-1* or of *TRK3* (see Discussion).

Since the initial kinetic studies were conducted with strain R3+NM, or cells derived from it, analysis of rubidium transport kinetics was repeated on cells with a 137c genetic background (Table 4). The kinetics of potassium/rubidium transport in wild-type *Chlamydomonas* change in response to the potassium concentration of the medium (Polley and Doctor 1985; Malhorta and Glass 1995). These previous studies demonstrated that when wild-type cells are starved of potassium for 18 h, the transport capacity (V_{max}) increases approximately 10-fold over that of cells grown in potassium-containing medium. Starved cells also exhibit an ability to discriminate between rubidium and potassium (Polley and Doctor 1985). This discrimination is indicated by an *increase* in the K_m for rubidium and a *decrease* in the K_m for potassium, i.e., starved cells have less affinity for rubidium and a greater affinity for potassium. The kinetic analyses of rubidium transport reported in this study are consistent with the earlier studies. In comparison with wild-type cells, the mutant clones exhibit altered kinetics when grown in potassium-replete medium; clones KDP3r and KDP5 have a reduced affinity for rubidium (a greater K_m), while clone KDP4 shows a V_{max} that is reduced by half. When starved of potassium, KDP4 exhibits "non-induced" kinetics, i.e. there is no increase in V_{max}; in contrast, the uptake of rubidium by KDP3r and KDP5 is so low that reliable kinetic analysis is not possible, at least by the methods used in this study.

Genetic evidence for a low-affinity potassium transport system

Plants and algae are known to possess two general types of potassium transport systems (Epstein 1973). The first is a high-affinity system that exhibits typical Michaelis-Menten kinetics. The second is a low-affinity system that exhibits apparently non-saturating or linear kinetics, and is believed to be mediated by a potassium channel (Kochian and Lucas 1988). Malhorta and Glass (1995) recently reported evidence from kinetic studies for a non-saturating potassium transport system in *Chlamydomonas* that operates at potassium concentrations higher than 1 mM.

In order to obtain additional insight into the nature of the low-affinity transport system, mutagenesis screens were performed to identify genes that encode this activity. It was initially assumed that mutant clones defective for a low-affinity transport system would exhibit a greater dependence on potassium (i.e., require more than 1 mM KCl) than the *trk* mutant clones, and that

Table 2 Growth rates in 0.1K0N medium of diploid cells bearing different combinations of *trk* mutations

Allele	Growth rate[a]				
	trk1	*trk2-1*	*trk2-2*	*trk3*	*TRK*
trk1	n.d.	7.3±1	8.1±0.4	8.1±0.4	7.4±0.3
trk2-1		2.0±1	1.9±0.6	8.0±0.4	8.0±0.5
trk2-2			0.0	7.6±0.1	7.7±0.4
trk3				0.0	7.8±0.4
TRK					7.7±0.2

[a] The growth rates of cells grown in 0.1 mM KCl are expressed as in Table 1. The values shown are the means and standard errors of three experiments. n.d., not determined

Table 3 Tetrad analysis of crosses

Cross	Alleles[a]								
	Linkage group II					Linkage group III			Linkage group X
	pf18	*nic2*	*act1*	*ac12*	*pf12*	*ac17*	*pf15*	*y7*	*nic23*
DP2×CC1715	55:0:8	55:0:9	36:0:27						
KDP5×CC1020				25:0:26	34:0:18				
KDP5×CC1680			16:0:12						
KDP3r×CC975						29:0:42			9:16:46
KDP3r×CC258							29:0:4		
KDP3r×CC1737						12:0:16		12:0:12	
KDP4×CC975						18:16:16			25:1:26

[a] The data are presented as parental ditype (PD):nonparental ditype (NPD):tetratype(T). Linkage is established when PD exceeds NPD and map distances were determined using the formula 6NPD×T

expression of this phenotype would be possible only in a *trk* background (i.e. in a genetic background defective for high-affinity transport).

Cells of strain DP2 were mutagenized by UV irradiation, and surviving cells were plated on 10K0N and 50K0N. Approximately 1000 clones that grew on each medium were selected for screening and were replica plated onto 1K0N solidified with purified agar. Of the 1000 clones from 10K0N, five failed to grow or grew poorly on 1K0N. Of the 1000 clones from 50K0N, eight failed to grow or grew poorly. One mutant clone which exhibited the clearest dependence on potassium concentrations greater than 1 mM was selected from each screen. These clones were designated 10KDP1 and 50KDP1, respectively, and their growth rates as a function of potassium concentration are shown in Table 5. Clone 10KDP1 exhibits wild-type growth rates at potassium concentrations greater than 5 mM; this requirement is specific for potassium and cannot be supplied by sodium (data not shown). The phenotype of 50KDP1 is, by contrast, more complex. While it requires potassium concentrations greater than 10 mM in order to grow, this requirement may be partially fulfilled by medium of high osmolarity, and it appears, therefore, to be a mutant clone that is defective in some aspect of osmoregulation.

Clone 10KDP1 was crossed to wild type in order to map the *hkr1* (high K^+ requiring) allele relative to the *trk2-1* allele. The four possible genotypes produced by this cross, however, resulted in only three phenotypes. Cells that grew on 0K0N medium solidified with purified agar and supplemented with 10 μM KCl were defined as having a low potassium phenotype. Cells that grew on plates containing 1 mM KCl were defined as being of intermediate potassium phenotype; whereas cells that grew on 10 mM KCl plates only were defined as high potassium phenotype. Of the four progeny genotypes, only the phenotype of the recombinant *TRK2-1 hkr1* was not established from previous experimental work (see Table 6). It was concluded, however, that this recombinant must have the low potassium phenotype because in tetrads that exhibit all three phenotypes among the progeny, there were twice as many progeny with the low potassium phenotype as there were with the high or intermediate potassium phenotype. These data indicate that the *TRK2-1* allele is epistatic to *hkr1*, and tetrad analysis shows that the *hrk1* is inherited as a simple Mendelian locus that assorts independently of the *trk2-1* locus (see Table 6). Efforts are currently underway to map the *HKR1* locus.

Crosses between 50KDP1 and wild type have to date resulted in few viable zygotes, and those that did germinate did not produce complete tetrads. The exact nature of the 50KDP1 phenotype and the position of its mutant allele await further analysis.

Table 4 Kinetics of ^{86}Rb uptake

Clone	Allele	Non-starved cells[a]			K-starved cells[a]		
		K_m	V_{max}	r^2	K_m	V_{max}	r^2
CC125	*TRK*	0.59±0.1	26±1	0.93	1.6±0.2	200±12	0.95
KDP3r	*trk3*	2.8 ±0.3	36±8	0.64	n.m.		
KDP4	*trk1*	0.51±0.1	10±1	0.74	2.0±0.7	11±3	0.55
KDP5	*trk2-1*	2.8 ±0.3	60±5	0.93	n.m.		

[a] The kinetic constants K_m (mM RbCl) and V_{max} (nmol Rb/h–10^6 cells) are shown for wild-type and mutant clones grown in 10K0N (non-starved cells) and for cells shifted at mid to late log to 0K0N and grown for 18 h (K-starved cells). The uptake rates are averages for four experiments in the case of CC125 and for two experiments for the each of the mutant clones. The kinetic constants and their standard errors were determined by linear regression of plots of V against V/S (Neame and Richards 1972). n.m., not measurable

Table 5 Growth rates of clones 10KDP1 and 50KDP1

Clone	Alleles	Growth rate[a]					
		50 mM KCl	25 mM KCl	10 mM KCl	5 mM KCl	1 mM KCl	10 mM KCl + 40 mM glucose
DP2	*trk2-1 HKR1*	7.6 ± 0.2	n.d.	8.0 ± 0.3	n.d.	7.0 ± 0.1	7.1 ± 0.3
10KDP1	*trk2-1 hkr1*	6.2 ± 0.3	n.d.	7.0 ± 0.3	5.2 ± 0.6	0.0	n.d.
50KDP1	*trk2-1 ?*	7.3 ± 0.1	5.6 ± 0.6	1.7 ± 1.3	0.0	0.0	4.8 ± 0.2

[a] The growth rate is expressed as described in Table 1. The values shown are the means and standard errors of three experiments. n.d., not determined

Discussion

The genetic evidence presented here supports the conclusion that *Chlamydomonas* possesses two distinct potassium transport systems – a high-affinity and a low-affinity system. The high-affinity transport system exhibits typical Michaelis-Menten kinetics and increases both its V_{max} and K_m for rubidium when cells are grown in medium depleted of potassium (Polley and Doctor 1985; Malhorta and Glass 1995). The kinetic constants reported in this study, however, are meaningful only as comparisons between mutant clones and are not necessarily an accurate measure of the physical properties of the potassium transporter. The reasons for this caveat are the ability of cells to discriminate between rubidium and potassium and the different transport kinetics obtained with different methods of measuring rubidium uptake (see below). Mutant clones, defective for a high-affinity potassium transport system were identified by their dependence on potassium concentrations higher than 0.1 mM for growth and their altered transport kinetics. Genetic analysis of the mutations indicates that three unlinked genes, *trk1*, *trk2* and *trk3*, are involved in high-affinity transport. The phenotypes of the *trk2-1* and *trk3* mutant alleles are characterized by similar alterations in both uptake kinetics and growth rates at low potassium concentrations. This similarity and the nearly identical growth rates of the single mutant clones KDP 5 and KDP3r and the recombinant clone DP7 (*trk2-1 trk3*), support the conclusion that the protein products of *TRK2-1* and *TRK3* interact functionally. By contrast, the phenotype of the mutant *trk1* allele is distinctly different from that of the *trk2-1* and *trk3* mutant alleles. Cells possessing this allele have a reduced V_{max} for rubidium uptake, and their growth rate, while lower than wild type, is higher than that of either *trk2-1* or *trk3* cells at 0.05 mM KCl. Since V_{max} is a measure of both the intrinsic rate of transport and the number of transporters per cell, one possible interpretation of the reduced V_{max} in *trk1* cells is a down-regulation of high-affinity potassium transporter synthesis. This reduction in V_{max} would be manifested as a hypomorphic phenotype, i.e., cells survive, but grow very slowly in low potassium medium, as is the case for the *trk1* clone. The behavior of the recombinant clones DP3 (*trk1 trk2-1*) and DP5 (*trk1 trk3*) is consistent with this interpretation. Their phenotypes, as measured by gowth rates at different potassium concentrations, are nearly identical and represent a combination of the phenotypes of the *trk1* and *trk2-1*, and *trk1* and *trk3* alleles,respectively; i.e. there is a "down regulation" of *trk2-1* and *trk3* growth rates over a range of different potassium concentrations.

The existence in *Chlamydomonas* of a second, low-affinity system operating at external potassium concentrations greater than 1 mM was first inferred from transport kinetics by Malhorta and Glass (1995). A similar analysis of transport kinetics, but employing a different procedure for measuring Rb^+ uptake (this study and Polley and Doctor 1985) failed to show non-saturating kinetics characteristic of low-affinity transport systems. The reasons for this discrepancy are not clear; nevertheless, a mutant clone, 10KDP1, possessing

Table 6 Genetic analysis of 10KDP1

Phenotype[a]	Genotype	Strain
High potassium	*trk2-1 hkr1*	Parental (10KDP1)
Low potassium	*TRK2-1 HKR1*	Parental (CC125)
Intermediate potassium	*trk2-1 HKR1*	Recombinant[b]
Low potassium	*TRK2-1 hkr1*	Recombinant

[a] Progeny of a cross between 10KDP (*trk2-1 hkr1 act1*) and the wild-type strain CC125 (*TRK2-1 HKR1 ACT1*) were analyzed for their potassium transport phenotypes, and their genotypes were inferred (see text). Tetrad analysis of segregation of *trk2-1* with respect to *act1* (linkage group II) and *hkr1* yielded PD:NPD:T distributions of 16:0:5 and 8:5:8, respectively. The different classes of tetrads were determined by the ratio of growing to non-growing progeny on media containing different concentrations of potassium. PD clones give a 2:2 ratio on both 0.01K0N and 1K0N; NPD = 2:2 on 0.01K0N and 4:0 on 1K0N; T = 2:2 on 0.01K0N and 3:1 on 1K0N

[b] This genotype is the same as that of KDP5

a phenotype consistent with a defect in a low-affinity transport system was isolated from mutagenized *trk2-1* cells. This mutant clone was identified by its dependence on potassium concentrations greater than 1 mM for growth. The mutant allele that confers this phenotype, *hkr1*, is unlinked to the *trk2-1* allele, and although *hkr1* has not yet been mapped, it seems unlikely that it is an allele of one of the other two *TRK* genes. Two considerations support this inference. First, genetic recombinants harboring two mutant *TRK* alleles do not exhibit a requirement for potassium concentrations greater than 1 mM in order to grow. By contrast, the *hkr1* allele in a *trk2-1* genetic background is associated with a requirement for greater than 5 mM potassium in order to achieve wild-type growth rates. The *hkr1* allele must, therefore, affect the function or regulation of some other transport system. Second – and consistent with the first reason, is the observation that *TRK2-1* is epistatic to *hkr1*. Cells that are defective for the low-affinity transport system (*HKR*) would still be able to grow as long as they possessed a functional high-affinity transport system (*TRK*), and in fact, *TRK2-1 hkr1* cells grow on medium with low concentrations of potassium.

In summary, mutant clones of *C. reinhardtii* that are defective in potassium transport were isolated and characterized genetically. Three genes, *TRK1*, *TRK2* and *TRK3*, affecting high-affinity potassium transport were identified. Genetic evidence suggests that the protein products of *TRK2* and *TRK3* interact to form the potassium transporter, while *TRK1* appears to regulate their expression or function. A fourth gene, *HKR1*, codes for a low-affinity transport system and the phenotype of its mutant allele is expressed in *trk2* background.

References

Anderson JA, Huprikar SS, Kochian LV, Lucas WJ, Gaber RF (1992) Functional expression of a probable *Arabidopsis thaliana* potassium channel in *S. cerevisiae*. Proc Natl Acad Sci USA 89:3736–3740

Cao Y, Ward JM, Kelly WB, Ichida AM, Gaber RF, Anderson JA, Uozumi N, Schroeder JI, Crawford NM (1995) Multiple genes, tissue specificity, and expression-dependent modulation contribute to the functional diversity of potassium channels in *Arabidopsis thaliana*. Plant Physiol 109:1093–1106

Epstein E (1973) Mechanisms of ion transport through plant cell membranes. Int Rev Cytol 34:123–168

Fu H-H, Luan S (1998) AtKUP1: a dual-affinity K^+ transporter from *Arabidopsis*. Plant Cell 10:63–73

Gassman W, Schroeder JI (1994) Inward rectifying K^+ channel in root hairs of wheat. Plant Physiol 105:1399–1408

Harris EH (1989) The *Chlamydomonas* sourcebook. Academic Press, San Diego

Kim EJ, Kwak JM, Uozumi N, Schroeder JI (1998) *AtKUP1*: an *Arabidopsis* gene encoding high-affinity potassium transport activity. Plant Cell 10:51–63

Kochian LV, Lucas WJ (1988) Potassium transport in roots. Adv Bot Res 15:93–178

Leigh RA, Wyn-Jones RG (1984) A hypothesis relating critical potassium concentrations for growth to the distribution and functions of this ion in the plant cell. New Phytol 97:1–13

Maathuis FJM, Sanders D (1994) Mechanism of high-affinity potassium uptake in roots of *Arabidopsis thaliana*. Proc Natl Acad Sci USA 91:9272–9276

Maathuis FJM, Sanders D (1997) Regulation of K^+ absorption in plant root cells by external K^+: interplay of different plasma membrane K^+ transporters. J Exp Bot 48:451–458

Malhorta B, Glass ADM (1995) Potassium fluxes in *Chlamydomonas reinhardtii* I. Kinetics and electrical potentials. Plant Physiol 108:187–197

Neame KD, Richards TG (1972) Elementary kinetics of membrane carrier transport. Blackwell, Oxford

Polley LD, Doctor DD (1985) Potassium transport in *Chlamydomonas reinhardtii*: isolation and characterization of transport deficient mutant strains. Planta 163:208–213

Rubio F, Gassmann W, Schroeder JI (1995) Sodium-driven potassium uptake by the plant potassium transporter HKT1 and mutations conferring salt tolerance. Science 270:1660–1663

Schachtman DP, Schroeder JI (1994) Structure and transport mechanism of a high-affinity potassium uptake transporter from higher plants. Nature 370:655–658

Schachtman DP, Schroeder JI, Lucas WJ, Anderson JA, Gaber RA (1992) Expression of an inward-rectifying potassium channel by the *Arabidopsis* KAT1 cDNA. Science 258:1654–1658

Sentenac H, Bonneaud N, Minet M, Lacroute F, Salmon J-M, Gaymard F (1992) Cloning and expression in yeast of a plant potassium ion transport system. Science 256:663–665

STIC-ILL

1/15/10

From: Lacourciere, Karen
Sent: Friday, May 10, 2002 3:43 PM
To: STIC-ILL
Subject: ill order

39583

Please provide the following references for use in examining 09/898,556

Mol. Cell. Biol. (1988), 8(8), 3104-13

Kagaku to Seibutsu (1995), 33(3), 146-8

Fudan Xuebao, Ziran Kexueban (2000), 39(6), 692-694

JOURNAL OF BACTERIOLOGY, (1994 Mar) 176 (5) 1488-99.

JOURNAL OF BACTERIOLOGY, (1996 Jan) 178 (2) 477-83.

GENE, (1998 Nov 5) 222 (1) 61-7.

MOLECULAR AND GENERAL GENETICS, (1999 Mar) 261 (2) 275-80.

ANIMAL GENETICS, (2001 Dec) 32 (6) 375-9.

Thank-you!

Karen A. Lacourciere Ph.D.
CM1 11D09 GAU 1635
(703) 308-7523

Asta

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

第39卷 第6期 复旦学报(自然科学版) Vol.39 No.6
2000年12月 Journal of Fudan University (Natural Science) Dec. 2000

文章编号:0427-7104(2000)06-0692-05

研究简报

锌指结构基因 *HKR1* 在脑胶质瘤中的表达分析

夏 放[1], 祁震宇[2], 戴建凉[1], 顾少华[1],
马良霄[1], 谢 毅[1], 毛裕民[1]

(1. 复旦大学 生命科学学院 遗传学研究所,上海 200433; 2. 苏州医学院,江苏 苏州 215007)

摘 要:从人胎脑cDNA文库中筛选到2 612 nt的锌指蛋白 *HKR1* 克隆,C端含有10个C_2H_2锌指结构,N端含有2个KRAB区域,基因芯片和原位杂交检测显示,*HKR1* 基因表达在脑胶质瘤中明显升高,提示该基因可能是一条潜在的肿瘤相关基因.

关键词:锌指蛋白;脑胶质瘤;基因芯片

中图分类号:Q 751 文献标识码:A

转录因子是具备调控DNA转录作用的DNA结合蛋白,通常含有能与DNA特异性结合的基元[1].锌指(Zinc finger, ZNF)结构是真核生物转录因子中最普遍的DNA连接基元之一,迄今已发现一千多个锌指蛋白(Zinc finger protein, ZNP),C_2H_2型是锌指中数量最多的一类,约占60%,其他还包括C_3HC_4型、C_4C_4型等. C_2H_2型锌指结构特征是在开放阅读框(ORF)C端存在高度保守的X_3-Cys-X_{2-4}-Cys-X_{12}-His-X_4-His(X:任意氨基酸)重复序列,通过2个半胱氨酸(Cys)和2个组氨酸(His)残基连接Zn^{2+},即C_2H_2-Zn^{2+}锌指[2];同时,近50%的C_2H_2型锌指蛋白ORF的N端含有保守的KRAB(Kruppel-associated box)区域[3],称为Kruppe Ⅰ型C_2H_2锌指蛋白,对DNA转录调控、信号转导起重要作用.

我们通过对人的18周胎脑cDNA文库进行大规模测序,筛选克隆到C_2H_2锌指蛋白HKP1(Human kruppel-related gene 1)[4]的cDNA序列,通过基因芯片和原位杂交技术对其在脑胶质瘤中的表达进行了初步分析.

1. 材料与方法

(1)cDNA文库的构建、测序及生物信息学分析 按文献[4]方法进行.

(2)基因芯片杂交 参见文献[5],其中2例原发人脑胶质瘤与1例正常脑组织标本由苏州医学院附属医院提供,抽取mRNA并反转录法标记探针. 杂交BioDoor™4000型基因表达谱芯片(UniGene),杂交参照说明书. 扫描芯片,分析的软件型号Cy3和Cy5荧光信号的强度和比值的判别标准同文献[5].

(3) 原位杂交 PCR法制备探针模板(3′-UTR,600 bp),T3 RNA聚合酶体外转录合成地高辛(DIG)标记的cRNA探针;7例原发人脑胶质瘤(男性,40~55岁)与1例正常脑组织标本由苏州医学院附属医院提供,经40 g/L多聚甲醛固定,液氮保存24 h后冰冻切片机连续冠状切片,0.4 g/L TritonX-100/0.1 mol/L PBS(pH7.2)和5 μg/mL蛋白酶K/0.1 mol/L PBS(pH7.2)37 ℃处理,在含DIG-cRNA探针2 μg/mL的杂交液中43 ℃杂交12~16 h,2×SSC冲洗30 min,碱性磷酸酶标记抗DIG抗体25 ℃孵育4 h,

收稿日期:2000-07-05
基金项目:上海市现代生物与新药产业发展基金资助项目(984319121)
作者简介:夏 放,(1969—),男,博士后.

NBT 和 BCIP 显色.

2. 结果和讨论

(1) *HKR*1 cDNA 序列的克隆与分析

从胎脑 cDNA 文库中经 Assembly 拼接获得一个 2 612 nt 的 cDNA 序列,通过 Blast 分析证实该序列即 C_2H_2 锌指蛋白 *HKR*1 的 cDNA 序列,该基因编码 640 个氨基酸,3′-UTR 为 643 nt. 在第 331～617 氨基酸编码区域有 10 个 C_2H_2 锌指结构(C-X_2-C-X_3-F-X_8-H-X_3-H),每个锌指结构共有 21 个氨基酸;锌指结构之间间隔 7 个氨基酸,为 SG-X-KP-X-V(H-C link). 在 N 端的第 65～130 氨基酸编码区域分别含有 A,B 2 个 KRAB(Kruppel-associated box)区域. *HKR*1 位于 19 号染色体 19q13.2,已发现有 15 个以上的 *ZFP* 基因存在于 19q13.2-13.3 区段,形成一个锌指结构密集区域,这些 *ZFP* 基因是否与 19p12 区有类似的进化机制,即可能在进化过程中由同一个"种子"*ZFP* 基因扩增而来[6],有待于作进一步相关分析.

(2) 基因芯片杂交结果分析

cDNA 基因芯片检测发现,在 2 例人脑胶质瘤标本中,*HKR*1 基因表达均明显高于正常脑组织(ratio 值:3.03; 2.40). 基因芯片是通过将大量的靶基因片段高密度有序地排列在硅制品载体上,既可以分析基因在各种组织细胞中的表达谱差异,也能对组织细胞的 RNA 作基因表达聚类分析,基因芯片表达谱技术分析近年来已被广泛用于基因功能研究. 脑胶质瘤是中枢神经系统最常见的恶性肿瘤,在颅内肿瘤中胶质瘤约占 40%,目前发现了近 100 种癌基因和 10 多种抑癌基因与胶质瘤密切相关[7]. 我们运用了基因芯片技术对人脑胶质瘤和正常成人脑组织作了表达谱分析,筛选出 10 多条与胶质瘤相关的基因,*HKR*1 基因是其中之一,提示该基因可能是一条潜在的癌基因.

(3) 原位杂交结果分析

原位杂交结果显示,DIG 标记的 *HKR*1 基因 cRNA 探针经在 7 例脑胶质瘤标本冰冻切片中杂交后,于大部分脑胶质瘤细胞中显色呈深蓝;而在正常成人脑组织中显色于少量大脑神经元细胞中,且着色较浅(见附页彩图 5),证实 *HKR*1 基因在人脑胶质瘤中表达高于正常脑组织. Kinzeler 等[8]于 1987 年发现了一条胶质瘤相关癌基因 glioma-associated oncogene(GLI),染色体定位在 12q13-q14.3,该基因在原发脑胶质瘤和细胞株中呈现高表达. 通过对核苷酸序列的分析,证实该基因含有 5 个与 DNA 结合的 C_2H_2 型锌指结构,在 N 端的氨基酸编码区域含有 KRAB 区域,Northern 分析发现 GLI 在胚胎内瘤细胞中表达而在大多数成人组织中不表达,*GLI* 与 *HKR*1 基因在脑胶质瘤发病机制中有无相互关联值得深入探讨. 研究表明,*HKR*1 基因在原发肺癌细胞中表达升高[4],肺癌细胞最易发生脑组织转移,而本实验所检测的 7 例脑胶质瘤标本均为原发灶,推测 *HKR*1 对于肺、脑组织的相关肿瘤研究有一定的参考价值.

参考文献:

[1] Evans R M, Hollenberg S M. Zinc finger: Gilt by association[J]. *Cell*, 1988, 52:1-3

[2] Liu C Z, Yang J T, Yoon J W, *et al*. Characterization of the promotor region and genomic organization of GLI, a member of the Sonic hedgehog-Patched signaling pathway [J]. *Gene*, 1998, 209: 1-11.

[3] Vissiing H, Meyer W K H. Repression of transcriptional activity by heterologous KRAB domains present in zinc finger proteins[J]. *FEBS Letters*, 1995, 369:153-157.

[4] Oguri T, Katoh O, Takahashi T, *et al*. The Krupple-type zine finger family gene, *HKR*1, is induced in lung cancer by exposure to platinum drugs[J]. *Gene*, 1998,222:61-67.

[5] 周宗祥,徐 坚,顾少华,等. *RBM*13 cDNA 的克隆及其表达谱分析[J]. 复旦大学(自然科学版),2000,39(6):583-587.

[6] Evan E, Susan M. Complex β-Satellite repeat structures and the expansion of the zinc finger gene cluster in 19q12[J]. *Genome Research*, 1998, 221:791-808.

[7] Kim I J, Lee K W, Park B Y, *et al*. Molecular cloning of multiple splicing variants of JIP-1 preferentially ex-

pressed in brain[J]. *J Neurochem*, 1999,72(4):1335-1343.

[8] Kinzler K W, Bigner S H, Bigner D D, *et al*. Identification of an amplified, highly expressed gene in a human glioma[J]. *Science*, 1987, 236:70-73.

Expression Analysis of Zinc Finger Protein HKR1 in Neuroglioma

XIA Fang[1], QI Zhen-yu[2], DAI Jian-liang[1], GU Shao-hua[1],
MA Liang-xiao[1], XIE Yi[1], MAO Yu-min[1]

(1. *Institute of Genetics, School of Life Sciences, Fudan University, Shanghai* 200433, *China*;
2. *Suzhou Medical College, Jiangsu Suzhou*, 215007, *China*)

Abstract: The cDNA of *HKR1*(Human kruppel-related gene 1)has been cloned from a human fetal brain cDNA library with 2 612 nt. It is indicated that HKR1 contains 10 highly conserved C_2H_2-type zinc finger motifs at the C-terminus and 2 KRAB(Kruppel-associated box)domains at the N-terminus. It has been found that the expression of HKR1 is higher in neuroglioma than those in normal brain tissue with cDNA microarray and *in situ* hybridyzation. The results suggest *HKR1* might be a putative cancer-associated gene.

Keywords: zinc finger protein; neuroglioma; microarray

征订简讯

上海邮政"185客户服务中心"已于2000年7月12日起开通.该"中心"受理的业务之一是接受用户电话订阅邮发报刊,用户可通过拨打"185"热线电话,就能方便地订阅所需要的邮发报刊.《复旦学报(自然科学版)》(双月刊)是邮局发行的期刊.本刊主要刊登复旦大学的自然科学和医学科学方面的学术论文,现还少许登载些博士生、硕士生及本科生的招生讯息.上海及全国各地的读者如需订阅本刊,也可拨打"185"热线电话予以征订.特此函告.

《复旦学报(自然科学版)》编辑部